FINAL REPORT

Study Title

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

Test Substances

B
L
U
F
Q
E
T
W

Reference Substance

H

Authors

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Study Completion Date

January 31, 2006

Performing Laboratory

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Study Number

05AE44-AE51, AE40.350064

Laboratory Project Number

4177

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STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity And Permeability Assay With Two Time Exposures and Optional Histology of the test substances, B, L, U, F, Q, E, T, W, and H, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test substances have not been determined by the testing facility.

John W. Harbell, Ph.D. Date
Study Director

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 05AE44-AE51, AE40.350064

Study Director: John Harbell, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	08-Sep-05	08-Sep-05	08-Sep-05
Initial Opacity Measurement	08-Sep-05	08-Sep-05	19-Sep-05
PAI – Microtomy	14-Dec-05	15-Dec-05	27-Dec-05
PAI – Quality Control	14-Dec-05	15-Dec-05	27-Dec-05
Histology Evaluation – Negative Control and 05AE47, 3 Minute Exposure Time	06-Jan-06	11-Jan-06	11-Jan-06
Final Report and Data	13-Jan-06	13-Jan-06	31-Jan-06

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

_____ _____

Amanda K. Ulrey, RQAP-GLP Date
Quality Assurance

SIGNATURE PAGE

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

Initiation Date:	September 7, 2005
Completion Date:	January 31, 2006
Sponsor:	
Sponsor's Representative:	
Testing Facility:	Institute for In Vitro Sciences, Inc. 21 Firstfield Road, Suite 220 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Study Director:	_____ John W. Harbell, Ph.D. Date
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Principle Investigator (slide preparation): Pathology Associates, A Charles River Company	David A. Hodge HT (ASCP)
Histological Evaluation performed by:	John W. Harbell, Ph.D.

TEST SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
05AE44	B	clear blue semi-viscous liquid	8/22/05	room temperature
05AE45	L	clear colorless non-viscous liquid	8/22/05	room temperature
05AE46	U	clear colorless non-viscous liquid	8/22/05	room temperature
05AE47	F	clear colorless non-viscous liquid	8/22/05	room temperature
05AE48	Q	clear colorless non-viscous liquid	8/22/05	room temperature
05AE49	E	clear light blue non-viscous liquid	8/22/05	room temperature
05AE50	T	clear colorless non-viscous liquid	8/22/05	room temperature
05AE51	W	clear colorless non-viscous liquid	8/22/05	room temperature
05AE40	H	clear colorless non-viscous liquid	8/1/05	room temperature

* - Protected from exposure to light

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test substances to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test substances. An *in vitro* score was determined for each of two exposure times tested for each test substance based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas.

The purpose of this study was to evaluate the potential ocular irritancy of the test substances as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. The laboratory phase of this study was conducted from September 8, 2005 to January 7, 2006 at the Institute for In Vitro Sciences, Inc. Three corneas were treated with each test substance at two exposure times of 3 and 10 minutes. Based on changes in corneal opacity and permeability (relative to the control corneas), an *in vitro* score was determined at each exposure time.

MATERIALS AND METHODS

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at 32 ± 1ºC for a minimum of 1 hour.

Assay Controls

The positive assay control used in this study was neat ethanol (Pharmco). The negative assay control used in this study was sterile, deionized water (Quality Biological).

Test Substance Preparation

As instructed by the Sponsor, each test substance was administered to the test system without dilution.

Test Substance pH Determination

The pH of each test substance was determined using pH paper (EMD Chemicals Inc./ EM Science). Initially, each test substance was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test substance was added to 0-6 pH, 5-10 pH, and/or 7.5-14 pH paper with 0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer. Three corneas, whose initial opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The treatment of each cornea was

identified with the test substance number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

Method for Testing Liquid or Surfactant Materials

The liquid test substances, B, L, U, F, Q, E, T, W, and H, were tested neat. An aliquot of 750 µL of the test substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. A set of three corneas was incubated in the presence of each test substance at 32 ± 1ºC for 3 minutes. A second set of three corneas was incubated in the presence of each test substance at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the negative control at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the positive control at 32 ± 1ºC for 10 minutes. After the 3 and 10-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test substances. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM (without phenol red) and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1ºC. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 µL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices *V*max kinetic microplate reader. If the OD_{490} value of a control or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 µL sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each comea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

As instructed by the Sponsor, histological evaluation was performed on corneas treated with F and H as well as the trial-matched control corneas. The fixed corneas were transferred to Pathology Associates, A Charles River Company (Frederick, MD) for embedding, sectioning and staining. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin

block so that a section of each half could be cut and placed on a single slide. Each slide was then stained with hematoxylin and eosin. Slides were returned to IIVS for evaluation.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD_{490} for the blank wells was calculated. The mean blank OD_{490} was then subtracted from the raw OD_{490} of each well (corrected OD_{490}). Any dilutions that were made to bring the OD_{490} readings into the linear range of the platereader (OD_{490} should be less than 1.500), had each diluted OD_{490} reading multiplied by the dilution factor. The final corrected OD_{490} of the test substances and the positive control was then calculated by subtracting the average corrected OD_{490} of the negative control corneas from the corrected OD_{490} value of each treated cornea:

Final Corrected OD_{490} = (raw OD_{490} – mean blank OD_{490}) – average corrected negative control OD_{490}

The mean OD_{490} value of each treatment group was calculated by averaging the final corrected OD_{490} values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test substance. Table 2 summarizes the opacity, permeability and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 40.1 to 65.0), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

The following classification system was established by Sina et al.[1] based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials.

In Vitro Score:

from 0 to 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

[1]Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score	pH
9/8/05	05AE44	B	Neat	3 minutes	90.0	1.201	108.0	10.0
				10 minutes	138.7	0.901	152.2	
	05AE45	L	Neat	3 minutes	2.0	0.052	2.8	8.0
				10 minutes	4.7	0.056	5.5	
	05AE46	U	Neat	3 minutes	2.7	0.014	2.9	9.0
				10 minutes	2.3	0.073	3.4	
	05AE47	F	Neat	3 minutes	14.0	0.281	18.2	0
				10 minutes	350.0	0.699	360.5	
	05AE48	Q	Neat	3 minutes	3.0	0.017	3.3	3.0
				10 minutes	9.0	0.299	13.5	
	05AE40	H*	Neat	3 minutes	0.0	0.139	2.1	11.5
				10 minutes	4.3	0.728	15.2	
9/12/05	05AE49	E	Neat	3 minutes	89.7	1.390	110.5	14.0
				10 minutes	169.3	1.793	196.2	
	05AE50	T	Neat	3 minutes	0.0	0.002	0.0	2.0
				10 minutes	1.7	0.010	1.8	
	05AE51	W	Neat	3 minutes	2.7	0.055	3.5	8.5
				10 minutes	4.7	0.071	5.7	
	05AE40	H*	Neat	3 minutes	-1.0	0.231	2.5	11.5
				10 minutes	1.7	0.758	13.0	

* - Benchmark substance, tested in each assay for comparison.

Table 2
BCOP Results of the Positive Assay Control

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score
9/8/05	Ethanol	10 minutes	28.0	1.066	44.0
9/12/05	Ethanol	10 minutes	26.7	1.320	46.5

Histological Evaluation

Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen in the cornea. This is not surprising as the test materials are applied topically to this “unprotected” epithelium. Each “layer” of the epithelium is scored for cell loss or damage. Figure 1 shows the structure of the epithelium from a control cornea (not from this study). Changes to the surface epithelium (squamous and upper wing cell epithelium) in the BCOP assay are usually not predictive of

lasting corneal changes *in vivo*. The loss of the squamous and upper wing cell layers in the excised bovine cornea by surfactant-based test substances appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo*. This type of damage is typically reversible in the rabbit. Many types of surfactants (e.g., sodium lauryl sulfate) are expected to lyse these cells so that they are progressively lost from the epithelial surface. The positive control, ethanol, provides an example of a different type of damage. In this case, the surface squamous epithelial cells are coagulated and fixed in place. The coagulated tissue stains heavily with eosin (see Figure 5). In addition, the wing and basal cells are vacuolated and the adhesion between the basal cells and the basal lamina is disrupted. Such changes can lead to sloughing of the epithelium. Lesions in the deep wing cell and basal cell layers, either by cell lysis or coagulation, are associated with more damage *in vivo*. Much of the available data come from studies on surfactants and show that loss of the bovine corneal epithelium is predictive of some corneal opacity in the rabbit[2].

Special effort has been made to detect changes in the stromal elements of the corneas. Jester[3], Maurer[4,5] and others have shown for a range of chemical classes that depth of injury in the early hours after exposure can be predictive of the eventual degree and duration of the ocular lesions. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deep injury to the stroma has more serious consequences. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling within the stromal collagen matrix and loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected by the presence of vacuole-like "holes" in the organized collagen matrix. Their appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of these vacuoles may be seen in Figure 8 where the positive control exposure has induced some stromal swelling. The depth and degree of vacuolization can be indicative of the degree of injury to the cornea and/or penetration of the test substance into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix vacuolization (swelling). Viable epithelium or endothelium will not only retard entry of the water but actively transport it out of the stroma. As the viability of epithelium declines, both the barrier and transport capacities are reduced. Thus, the amount of water accumulating in the upper stroma reflects the damage to the epithelium. When the full epithelial layer is lost over a section of stroma, stromal swelling can increase the stromal thickness by 50% or more over the control corneal stroma. Such changes are often observed when corneas treated with severe irritants are incubated for extended periods after exposure. Loss of the endothelium can also lead to appreciable deep stromal swelling. The loss may result from test substance penetration or

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[2] Gettings, SD, Lordo, RA, Hintze, KL, Bagley, DM, Casterton, PL, Chudkowski, M, Curren, RD, Demetrulias, JL, DiPasquale, LC, Earl, LK, Feder, PI, Galli, CL, Glaza, SM, Gordon, VC, Janus, J, Kuntz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food Chemical Toxicology** 34:79-117.

[3] Jester, J.V., Li, H.F., Petroll, W.M., Parker, R.D., Cavanaugh, H.D., Carr, G.J., Smith, B., and Maurer, J.K. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Invest Ophthalmol Vis Sci** 39:922-936.

[4] Maurer, J.K. and Parker, R.D. (1996) Light microscopic comparison of surfactant-induced eye irritation in rabbits and rats at three hours and recovery/day 35. **Toxicologic Pathology** 24:403-411.

[5] Maurer, J.K., Parker, R.D., and Jester, J.V. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test substance exposure tends to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix vacuolization will be located in the deep stroma (just above Descemet's Membrane). In contrast, test substance-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix vacuolization will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

In vivo, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through "scar" collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test substance-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the beginning of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic eosinophilia. Harbell and Curren[6] have reported that mechanical removal of the corneal epithelium leads to marked stromal swelling (marked collagen matrix vacuolization). The keratocytes in the zone of marked swelling undergo changes as well. The nuclei become enlarged and the cytoplasmic staining is very eosinophilic. Thus, when test substance-treated corneas show only this type of keratocyte change (with stromal swelling), the change may be the result of the stromal swelling rather than direct action of the test substance on the cells.

Since depth of injury (both extracellular and cellular) to the stroma is important in the analysis, a means to describe the depth of injury is necessary as part of this analysis. Determining this depth is not always a straightforward process. Because of the topical application of the test substance to the epithelium, one would expect that exposure to the stroma would progress from the area just under Bowman's Layer down through the stroma to Descemet's Membrane. There is no external inflammatory process *in vitro*, so one might also expect the progression of damage to follow the progression of exposure. That means that damage to the stroma should first appear close to Bowman's Layer. As the damage to the stroma increases, deeper layers might be involved. This implies that one would want to express damage as progressing from the anterior (Bowman's Layer) to the posterior (Descemet's Membrane) and express it as a fraction of the total stromal depth involved. However, collagen matrix vacuolization can (and often does) increase total stromal thickness. Thus, measurements of the depth of a stromal lesion can be complicated by the change in overall stromal thickness. To account for stromal swelling, this depth is actually estimated from the percentage of the stromal cross section that remained undamaged (starting at the posterior border). For example, a cornea reported to show collagen matrix vacuolization to 30% depth would mean that 70% of the cross section of that cornea

[6] Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. **ALTEX** 42(Special Issue):236.

(starting at Descemet's Membrane) did not show vacuolization. For this report, depth of stromal damage is reported as the percentage of the normal corneal depth (cross-section) involved, starting from the anterior border (Bowman's Layer). It should be clearly understood that the percentage of the stromal depth is only an estimate developed by evaluating several fields in each cornea (where possible). The values are, by necessity, approximations of an average depth to which the lesion extended (e.g., collagen matrix vacuolization). It would be unwise to try to compare small differences in the reported depth. Rather, one should focus on broader bands of depth (e.g., upper, middle and deep stroma).

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Where little or no damage to the stroma was detected, the area directly under Bowman's Layer was photographed since that is where one would expect to see damage if it had occurred. Images were prepared using a Spot Insight Digital Camera and Spot 4.0.8 software (Diagnostic Instruments, Inc., Sterling Heights, MI). The color balance of the images was corrected to better represent the colors that would be seen through the microscope. Photomicrographs taken of the epithelium often overexposed the stroma. This leads to the impression that the stroma is more damaged (e.g., vacuolated) than it really is. Stromal changes are better represented in micrographs where the stroma is the central feature of the image.

With the digital camera and associated software, it is possible to measure distances within the captured image. This feature can be used to measure corneal thickness directly. Such measurements require a true cross-section of the cornea (i.e., a perpendicular section relative to the corneal surface plane). In some cases, a true cross-section is not available. The Descemet's Membrane thickness is used to estimate how close the section is to a true cross section. The appearance of an unusually thick membrane suggests that the section was cut at a tangent to the true cross section (or a very old animal). If the measured corneal section did not appear to be a true cross section, the observation was noted. Even in a true cross section, the isolated cornea is not of uniform thickness but rather shows peaks and valleys. Therefore, an effort has been made to select "representative" cross-sections that are neither extreme "peaks" nor "valleys" for measurement of corneal thickness. An example of such a measurement is seen in Figure 4. The values obtained should be considered "representative" of the treatment group rather than strict quantitative measures. Many more measurements would be required to provide a quantitative comparison. In some cases, the treated corneas will have lost most of their epithelium or it has been compromised (i.e., ethanol treatment). In those cases, the stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

Histological Evaluation

The negative control corneas were treated for 10 minutes with sterile, deionized water (slides B8496-B8498). The negative control-treated epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. The basal cells were always tightly attached to each other. Several layers of wing cells covered the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figure 2).

The stromal elements showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining to narrow, elongated and condensed

with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. Rare cells, with eosinophilic cytoplasmic staining, were observed. Collagen bundles were generally parallel and well ordered (Stroma just under Bowman's Layer, Figure 3).

The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well maintained.

A cross section of the negative control showing the general thickness of the whole cornea and stroma is provided in Figure 4.

The positive control corneas (slides B8499-B8501), treated for 10 minutes with 100% ethanol, showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei (Figure 5). The loss of adhesion between the basal cells (cell to cell) and the basal lamina was marked. The epithelium was probably not viable at the time of fixation. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 6). In the stroma directly below Bowman's Layer, the collagen matrix showed slight to moderate hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate/marked collagen matrix vacuolization extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 7) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization or other forms of abnormal nuclear condensation (Figure 8). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with larger nuclei and some cytoplasmic eosinophilia (Figure 9). The endothelial cells were generally intact (similar to the negative control-treated corneas).

Table 3
Histological Observations on the Test Substance -Treated Corneas

IIVS Number	Sponsor's Designation	Observations	Figure #
05AE47 Slides B8520-B8522	F, neat, 3-minute exposure, 120-minute post-exposure, 09/08/05	Epithelium: The full squamous cell layer was coagulated and the nuclei showed moderate blanching (loss of stainable components). The upper wing cells showed abnormally condensed nuclei, cytoplasmic vacuolization and a general degeneration in tissue structure. In some fields, the coagulated squamous cells had separated from the basal cells because of a complete breakdown of the upper wing cell layer. The basal cells were structurally intact but showed moderate cytoplasmic vacuolization. In some fields there was also separation between the basal cells and the basal lamina (Figures 10 and 11). Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 12). Slight to moderate collagen matrix vacuolization extended to through most of the stromal depth. There was a marked increase in	10 - 15

IIVS Number	Sponsor's Designation	Observations	Figure #
		the frequency of keratocytes with abnormal chromatin condensation (and some vacuolization) through most of the stromal depth (Figures 13 and 14). There was also slight to moderate increase in collagen matrix vacuolization directly above Descemet's Membrane suggesting that there was a degradation of endothelial cell function during the incubation (Figure 15). Endothelium: The endothelium was damaged in many fields and the overall increase in collagen matrix vacuolization directly above Descemet's Membrane suggested a degradation of endothelial cell function.	
05AE47 Slides B8523-B8525	F, neat, 10-minute exposure, 120-minute post-exposure, 09/08/05	Cornea #32 showed a modest opacity increase (22.7) but a high OD_{490} (2.053) while corneas #35 and #37 showed extreme opacity scores (513.7 each) and minimal OD_{490} values (0.040 and 0.001 respectively). The basis for these differences is the formation of gas pockets in the stroma of corneas #35 and #37. This type of lesion has been observed in some corneas treated with peroxide-containing formulations (Harbell, unpublished). Epithelium: The epithelium showed coagulation through all three layers. The coagulated epithelium had separated from the Bowman's Layer (Figure 16) (cornea #32). Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas. Cornea #32 was appreciably thicker (Figure 17) and corneas #35 and #37 were very much thicker (Figure 18, cornea #37). Cornea #32 showed moderate collagen matrix vacuolization through the full depth of the stroma. The collagen matrix directly above Descemet's Membrane showed increase vacuolization suggesting a degradation of endothelial cell function. There was a marked increase in the frequency of keratocytes with various degrees of hyper-condensation of their nuclei (and some full pyknosis) (Figures 19 and 20). Corneas #35 and #37 showed major gas vacuoles through much of their stromal depth. Figure 21 showed the interface between the collagen matrix directly below Bowman's Layer	16 - 21

IIVS Number	Sponsor's Designation	Observations	Figure #
		and zone containing the gas vacuoles (cornea #37). The failure of fluorescein to pass through the cornea can be attributed to these gas vacuoles. Endothelium: The endothelium was lost or showed degraded function on all three test substance-treated corneas.	
05AE40 Slides B8532- B8534	H, neat, 3-minute exposure, 120-minute post-exposure, 09/08/05	Epithelium: The full squamous cell layer was lost from most fields. The wing and basal cells were intact but showed a moderate increase in cytoplasmic vacuolization (Figure 22). Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 23). Marked to moderate collagen matrix vacuolization extended to mid depth and moderate vacuolization extended into the deep stroma. In some fields, there was increased vacuolization directly above Descemet's Membrane suggesting degradation of endothelial cell function. In the upper half of the stroma, there was a moderate increase in the frequency of keratocytes with slightly abnormal nuclear morphology and increase cytoplasmic eosinophilia (Figure 24). Endothelium: The slight increase in collagen matrix vacuolization directly above Descemet's Membrane and loss of endothelium in other fields suggest some degradation of endothelial cell function.	22 - 24
05AE40 Slides B8535- B8537	H, neat, 10-minute exposure, 120-minute post-exposure, 09/08/08	Epithelium: The squamous cell layer was lost in all sections. In many of fields, the wing and basal cells were present but showed abnormal nuclear condensation and cytoplasmic vacuolization. In many other fields, the basal cells showed marked abnormal nuclear condensation and a breakdown of the cells between the wing and basal layers (Figures 25 and 26). In some cases, the basal cells had separated from the basal lamina. Bowman's Layer was intact. Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 27). Moderate collagen matrix vacuolization extended through most of the stromal depth. As with the 3-minute treatment group, there was a slight but distinct increase in	25 - 29

IIVS Number	Sponsor's Designation	Observations	Figure #
		collagen matrix vacuolization directly above Descemet's Membrane. The keratocyte changes were striking. Through most of the stromal depth, the keratocytes showed a marked increase in the frequency of cells with hyper-condensation of their nuclei (but without appreciable cytoplasmic eosinophilia) (Figures 28 and 29). This pattern of keratocyte changes is consistent with exposure to a reactive chemical. Endothelium: The endothelium was damaged in many fields and the increased collagen vacuolization suggests degradation in endothelial cell function.	

The figures displayed on the subsequent pages of this report are representative hematoxylin and eosin-stained cross-sections presented at the indicated magnification. The black bar, on each micrograph, represents 100 µm. Arrows from the text to the figures are intended to show examples of the lesions mentioned. Not all lesions are marked. The vertical double-headed arrows mark the limits of the epithelium in the appropriate figures.

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (magnification 290x)

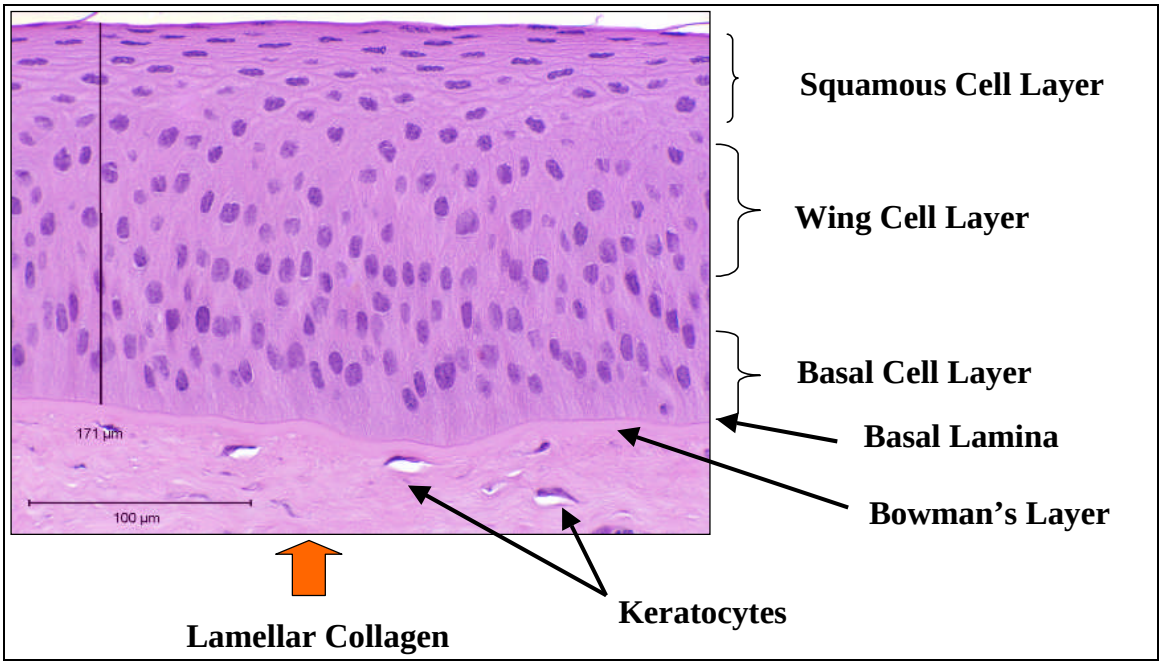


Figure 2. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (magnification 237x)

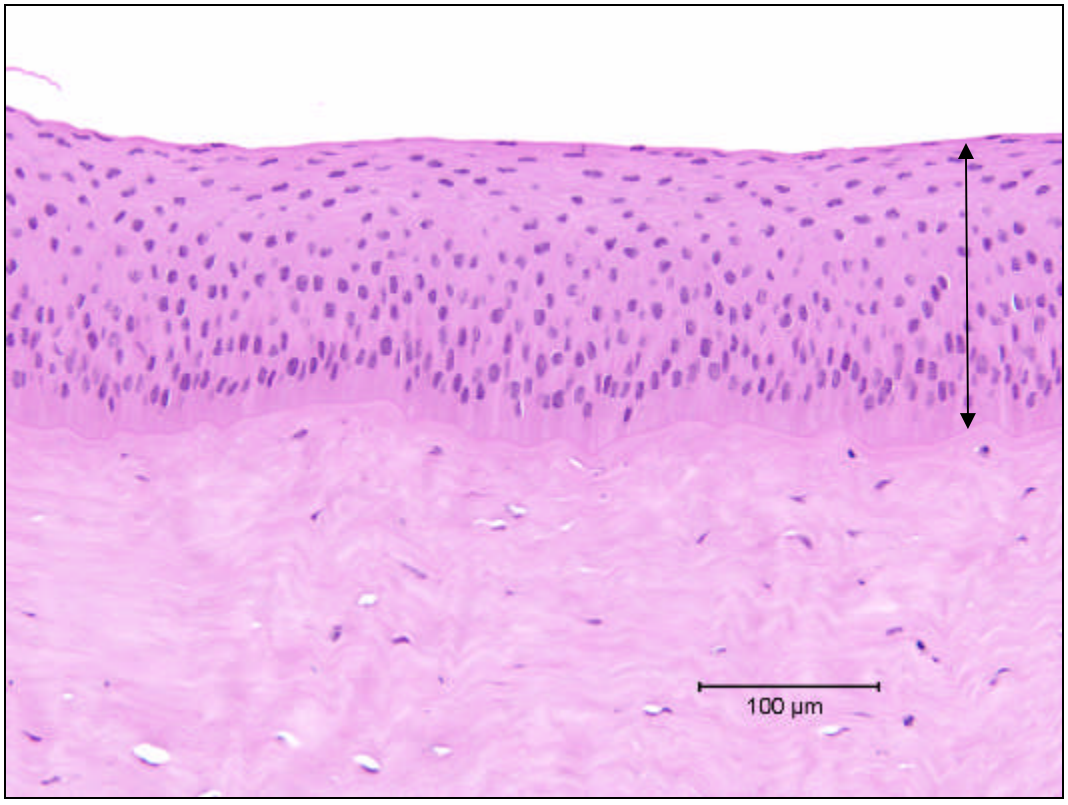


Figure 3. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma directly below Bowman's Layer (magnification 475x)

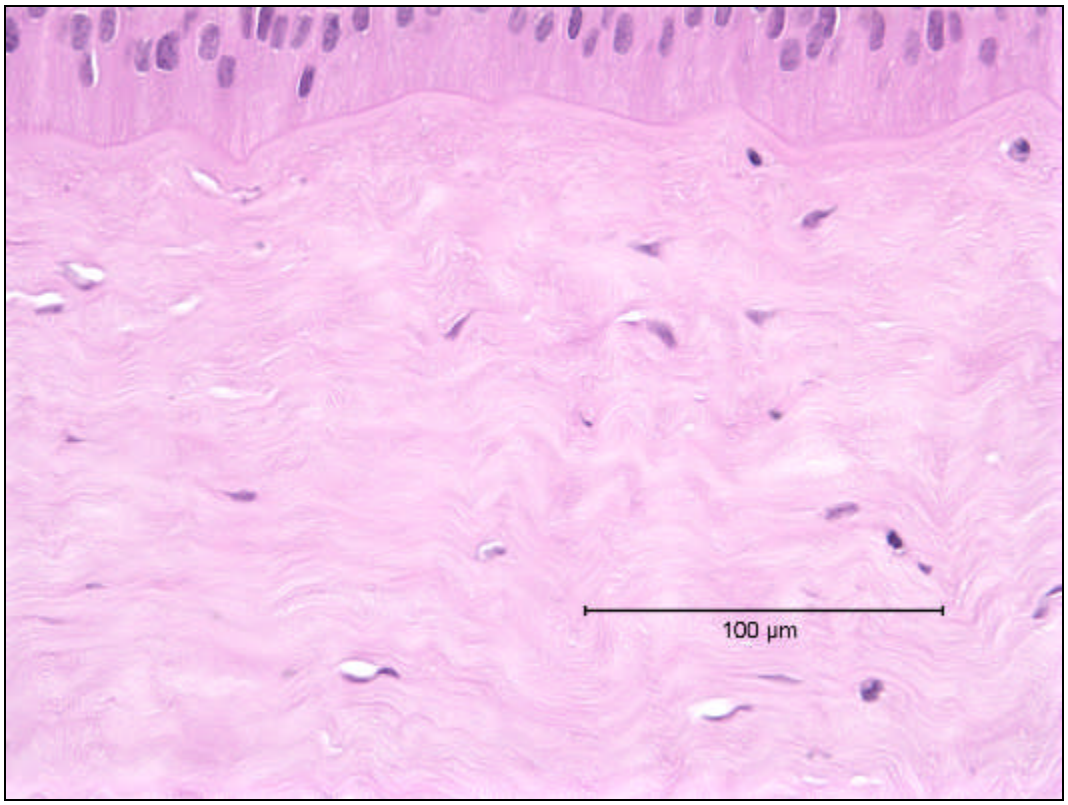


Figure 4. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

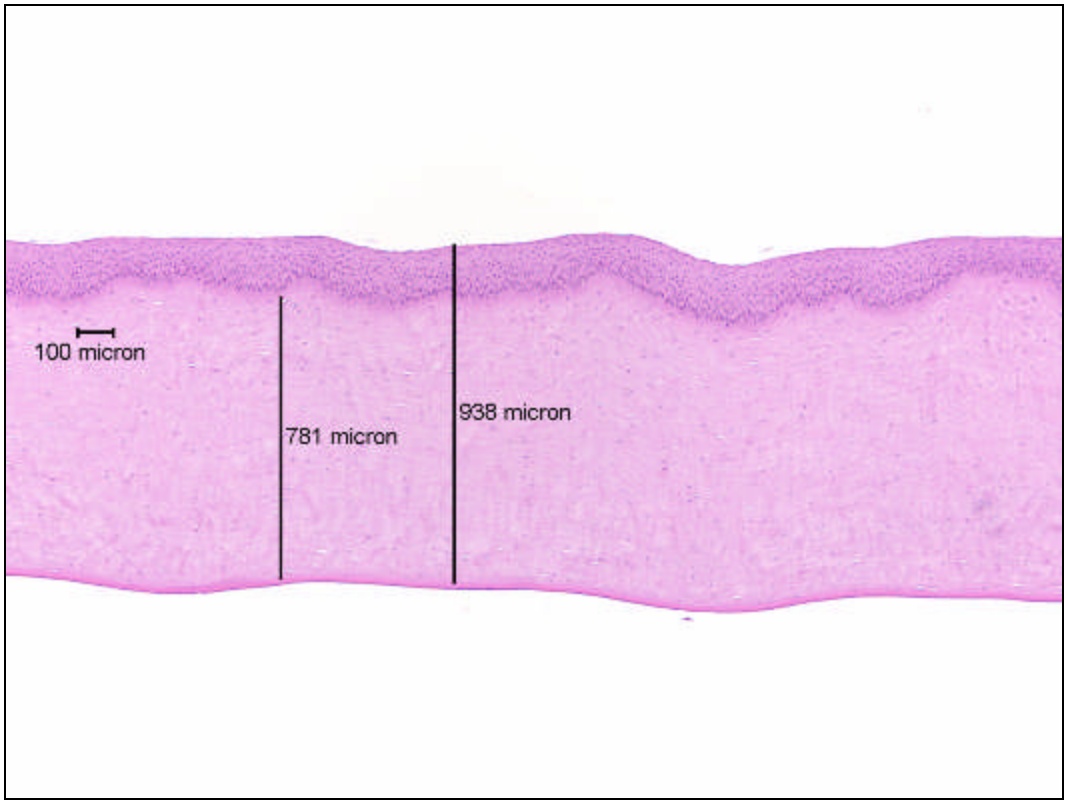


Figure 5. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (probably not viable at the time of fixation) (magnification 237x)

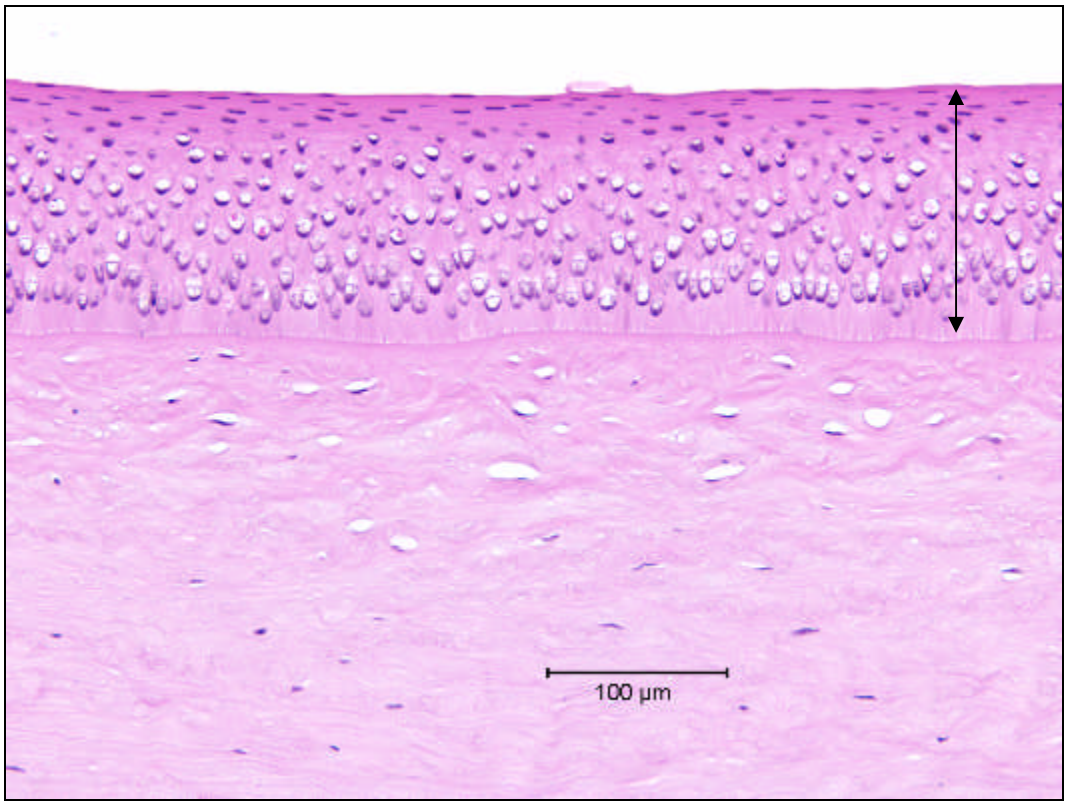


Figure 6. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

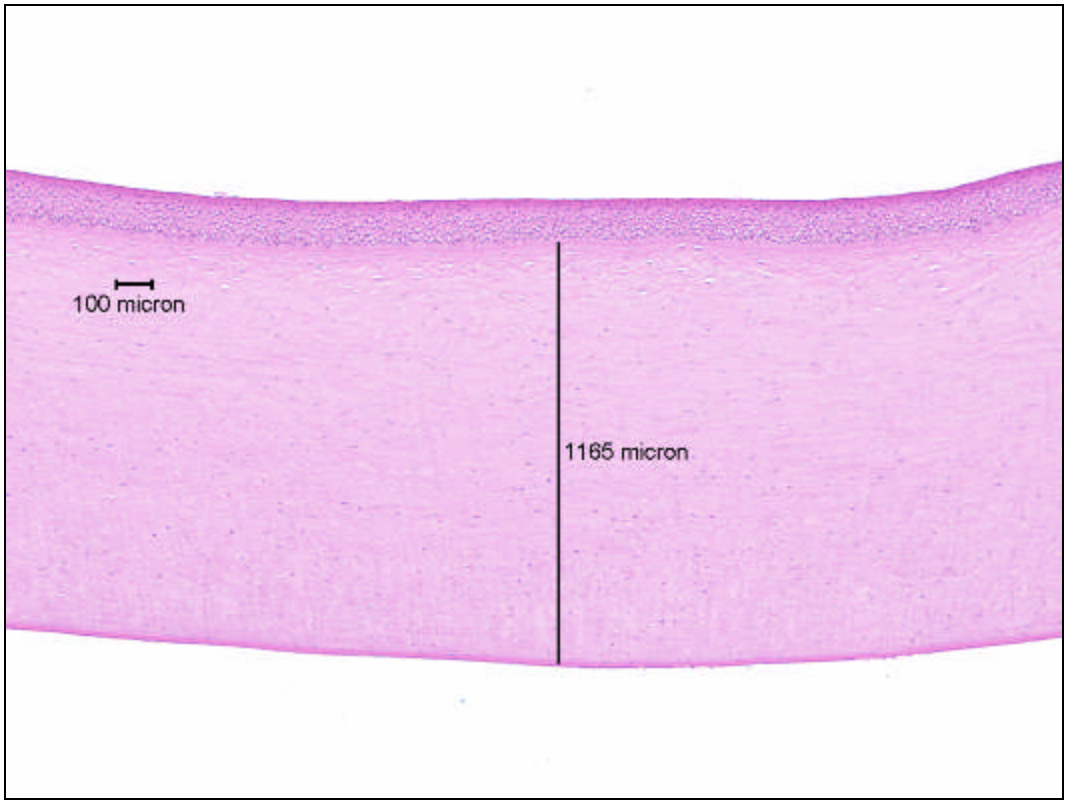


Figure 7. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Upper stroma showing hyperchromic staining in the zone directly below Bowman's Layer and the decrease in the density of viable keratocytes (magnification 237x)

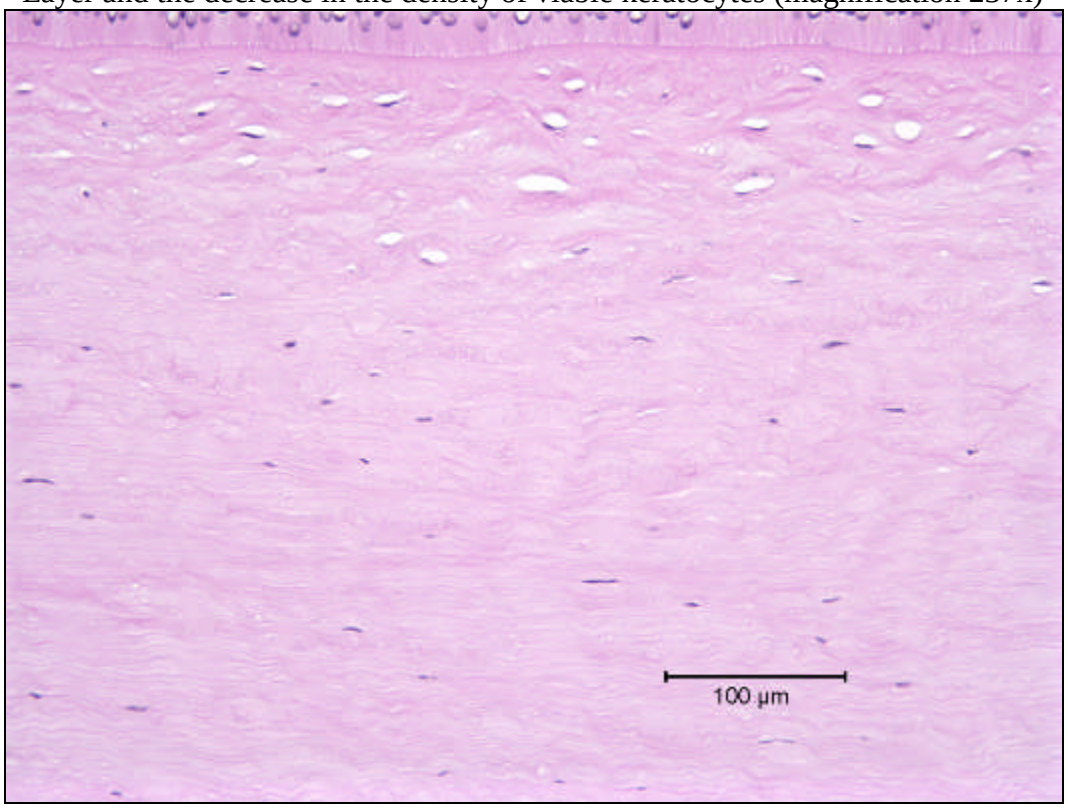


Figure 8. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and an increased frequency of keratocytes with abnormal chromatin condensation (magnification 475x)

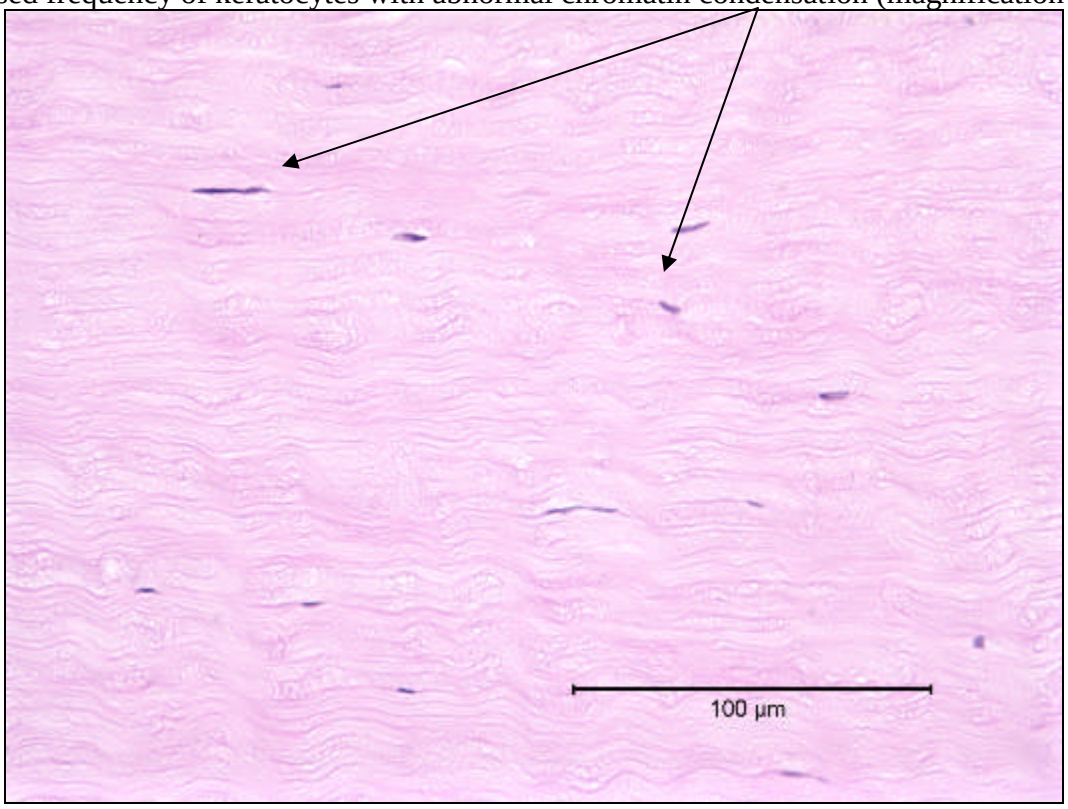


Figure 9. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma below mid depth showing keratocyte with larger nuclei and some cytoplasmic eosinophilia (magnification 475x)

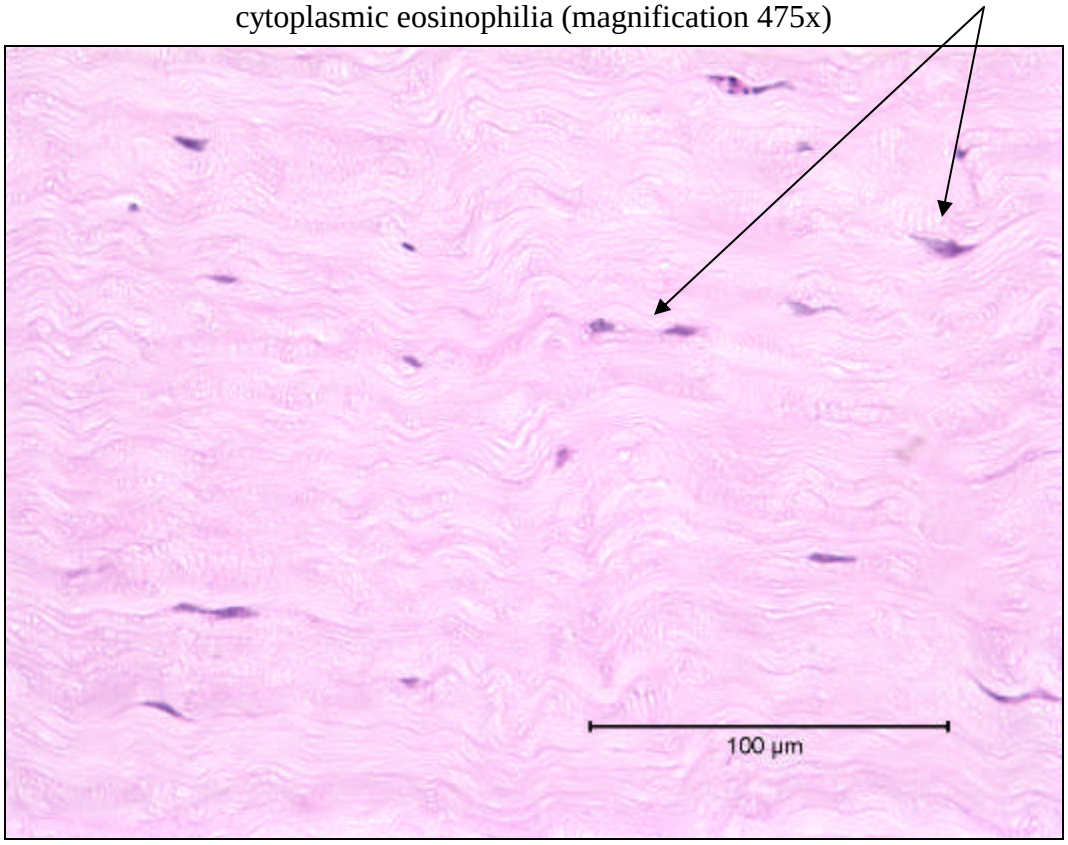


Figure 10. F, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Epithelium (overview) (magnification 237x)

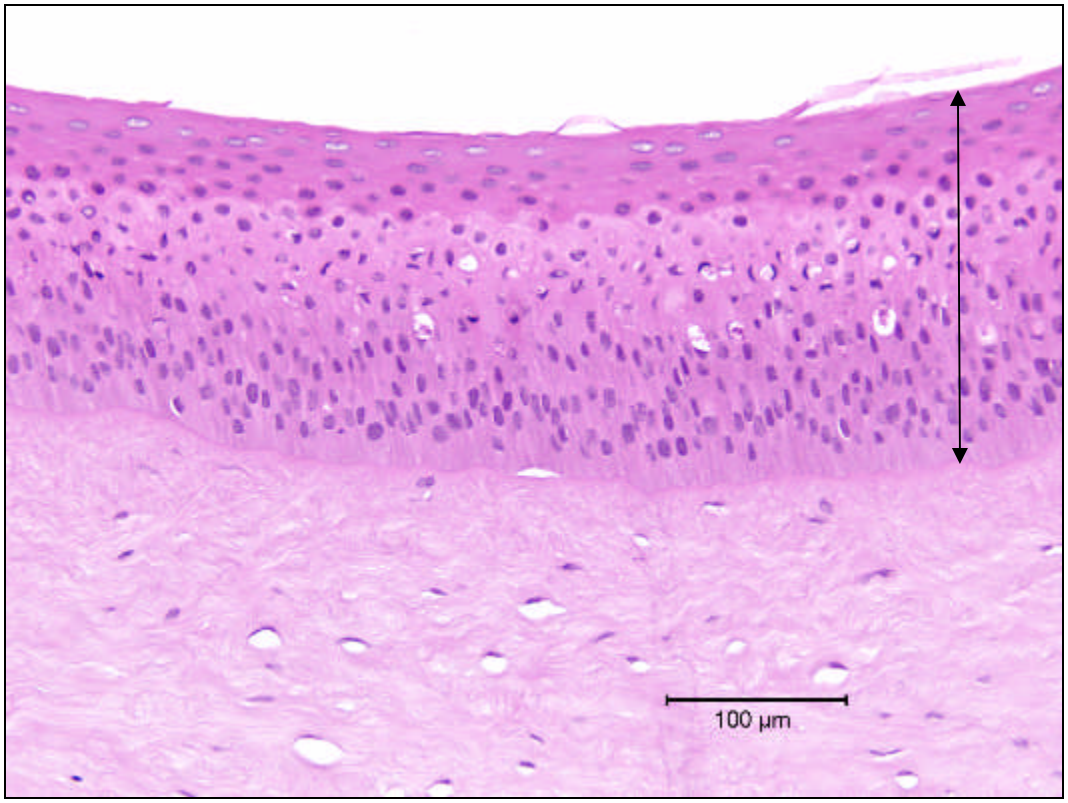


Figure 11. F, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Epithelium showing coagulation of the squamous cells, nuclear condensation and cytoplasmic vacuolization in the upper wing cells and cytoplasmic vacuolization in the basal cells (magnification 475x)

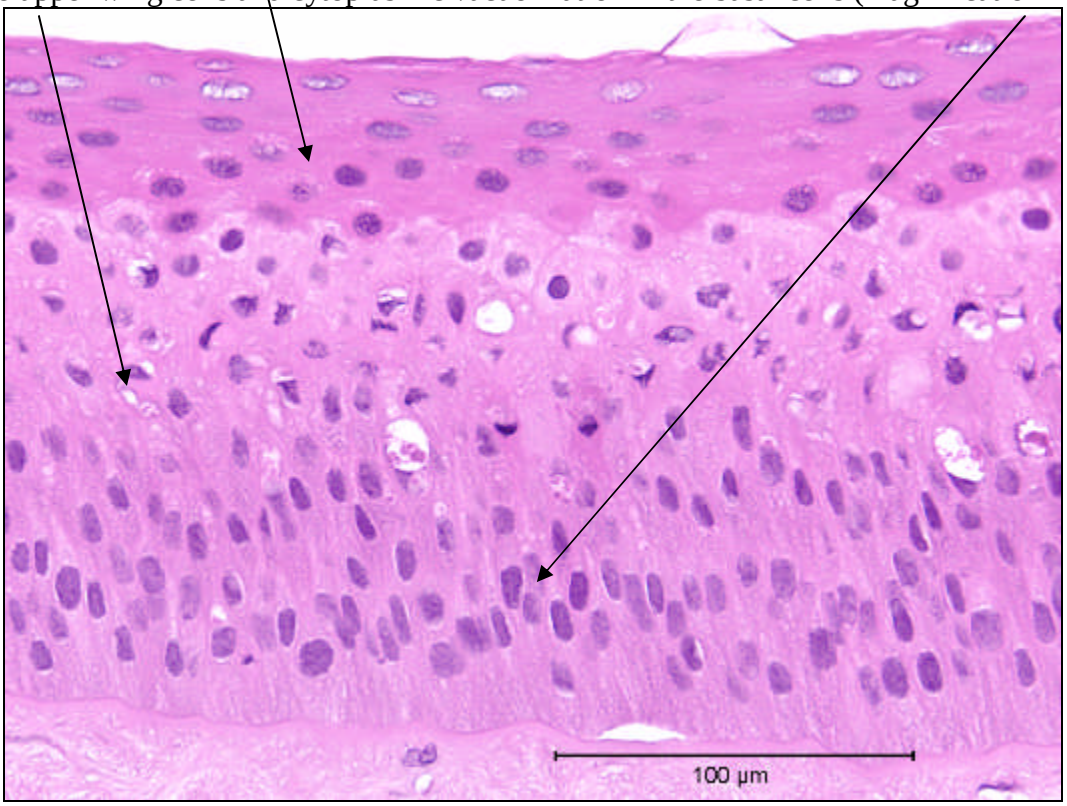


Figure 12. F, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Full thickness (magnification 48x)

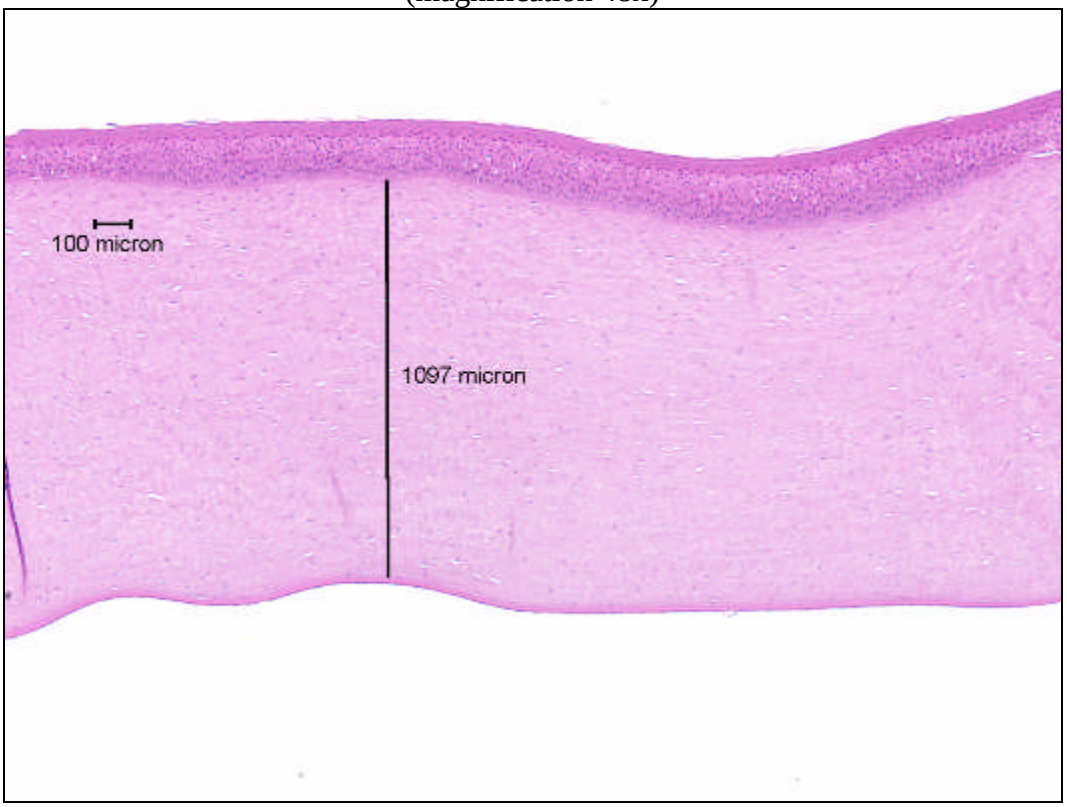


Figure 13. F, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with abnormal chromatin condensation (magnification 475x)

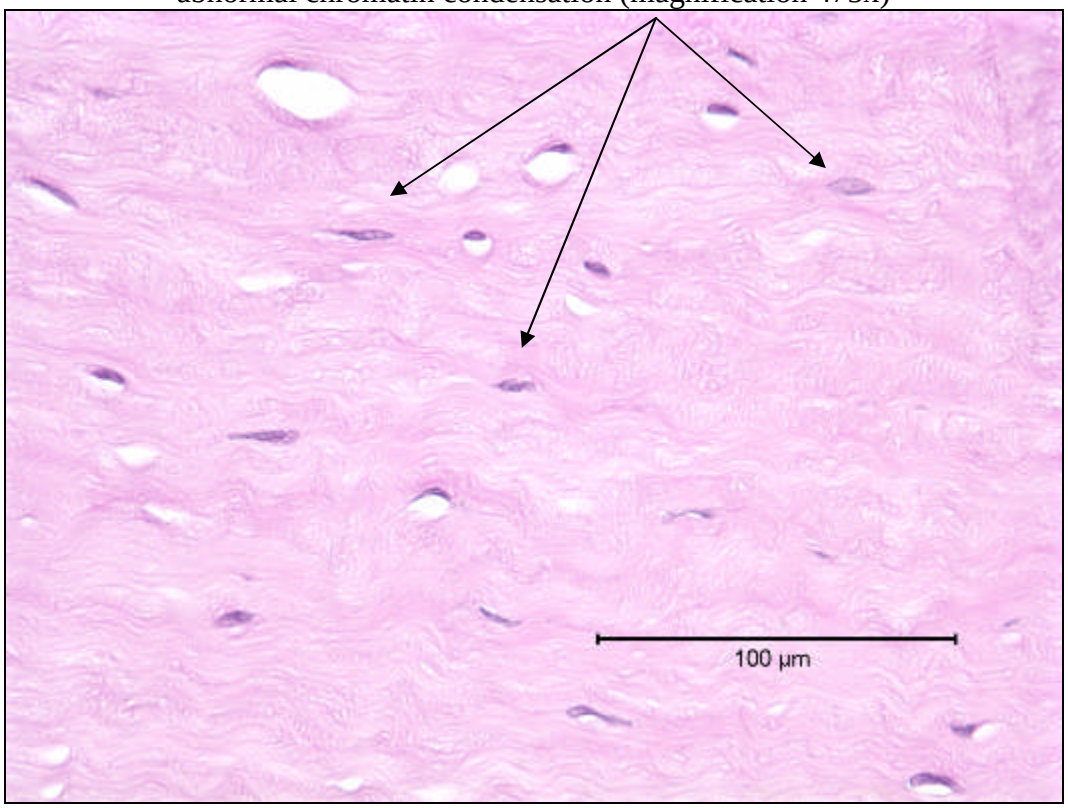


Figure 14. F, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Stroma at mid depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with abnormal chromatin condensation (magnification 237x)

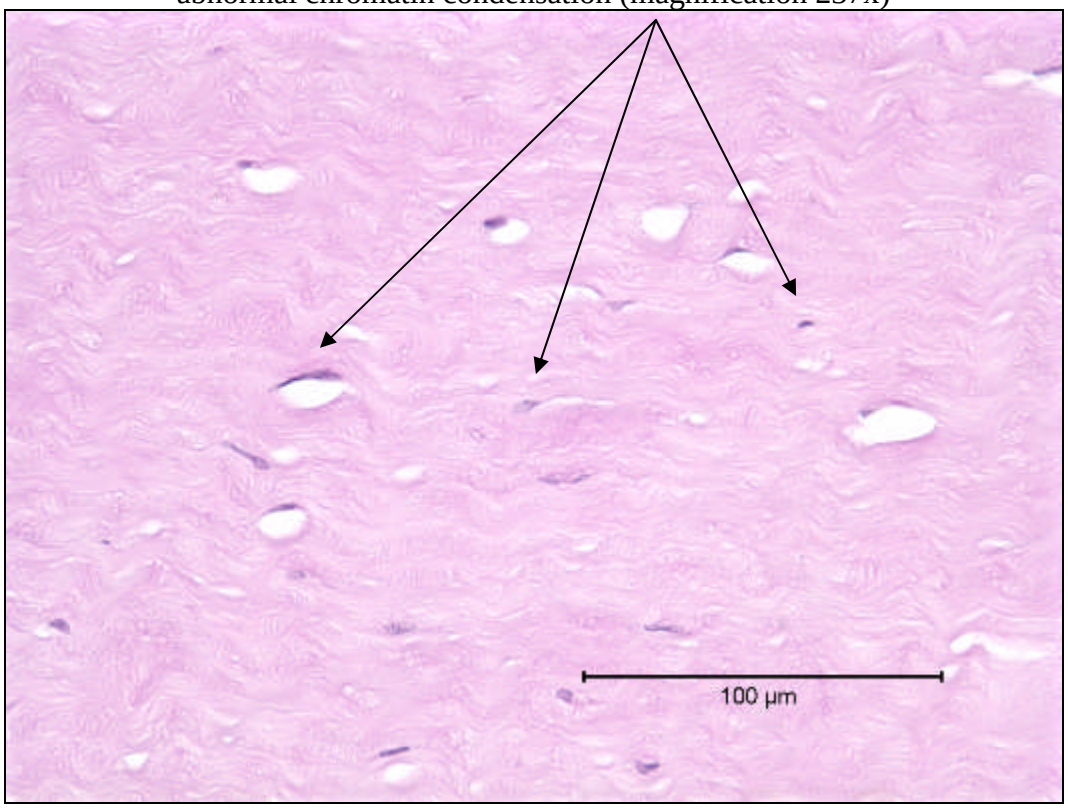


Figure 15. F, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Stroma at just above Descemet's Membrane showing slight to moderate collagen matrix vacuolization and an increase in keratocytes with abnormal chromatin condensation (magnification 475x)

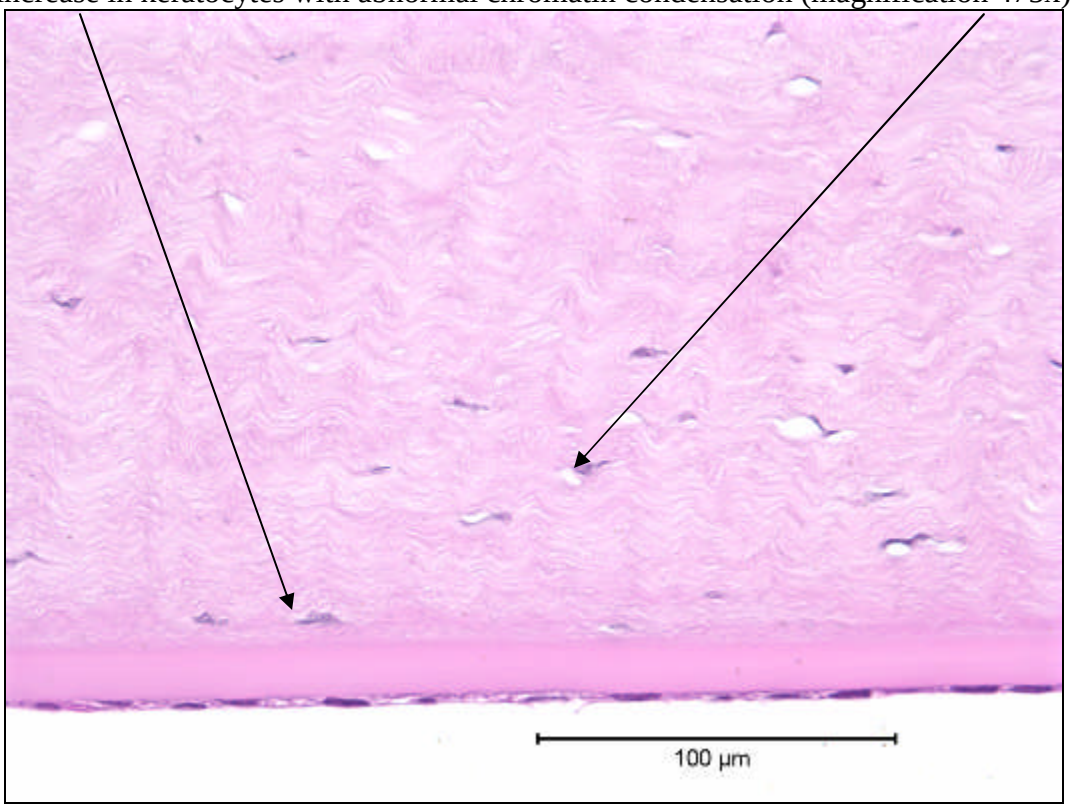


Figure 16. F, neat, 10-minute exposure, 120-minute post-exposure (09/08/05)(cornea #32) - Epithelium (nonviable at the time of fixation) (magnification 237x)

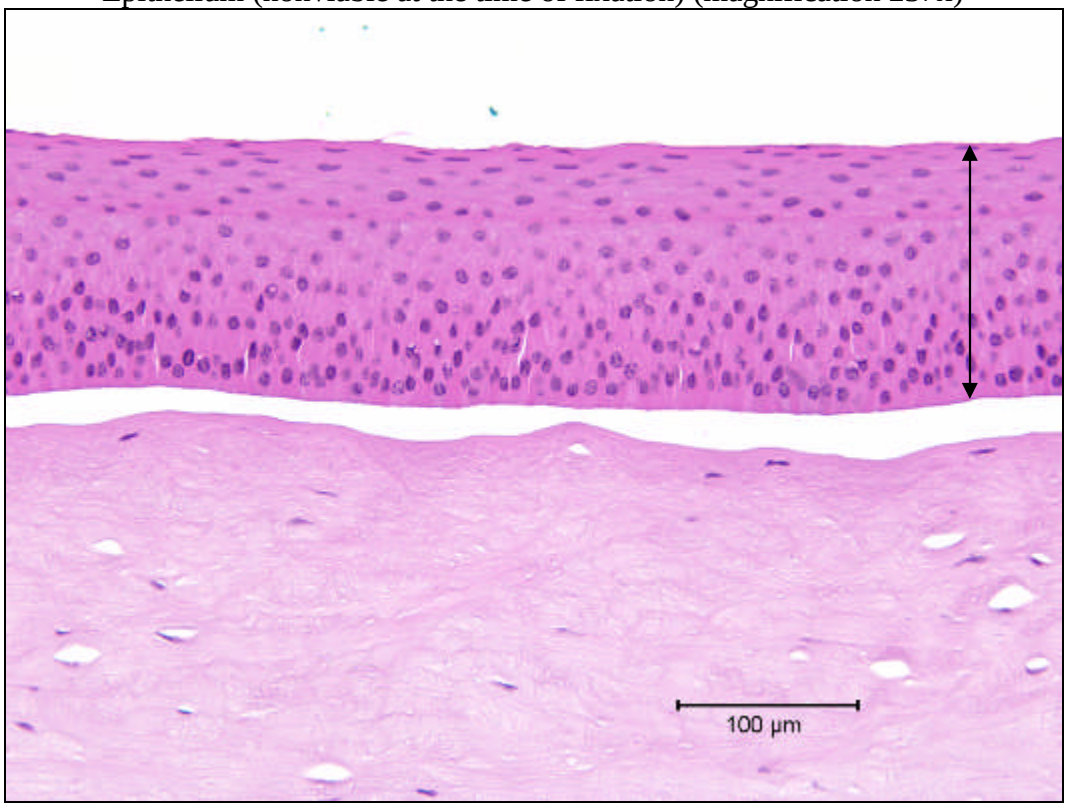


Figure 17. F, neat, 10-minute exposure, 120-minute post-exposure (09/08/05)(cornea #32) - Full thickness (magnification 48x)

Figure 18. F, neat, 10-minute exposure, 120-minute post-exposure (09/08/05)(cornea #37) - Full thickness (magnification 48x)

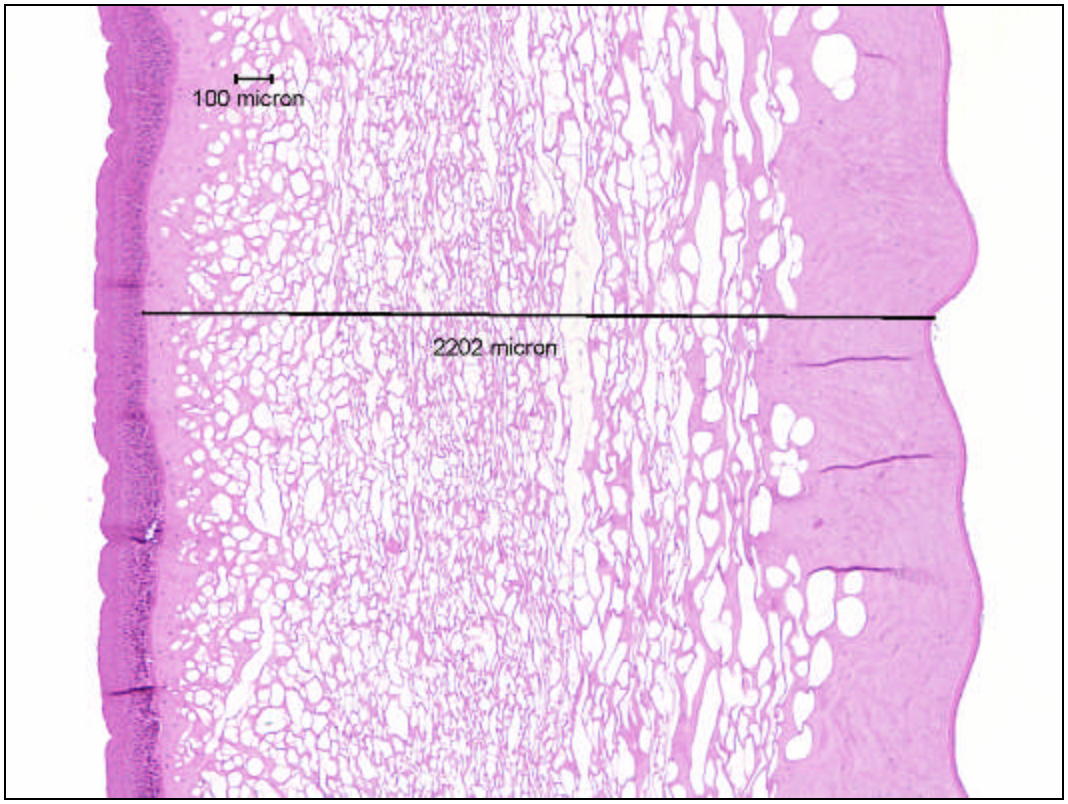


Figure 19. F, neat, 10-minute exposure, 120-minute post-exposure (09/08/05)(cornea #32) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with hyper-condensed nuclei (magnification 475x)

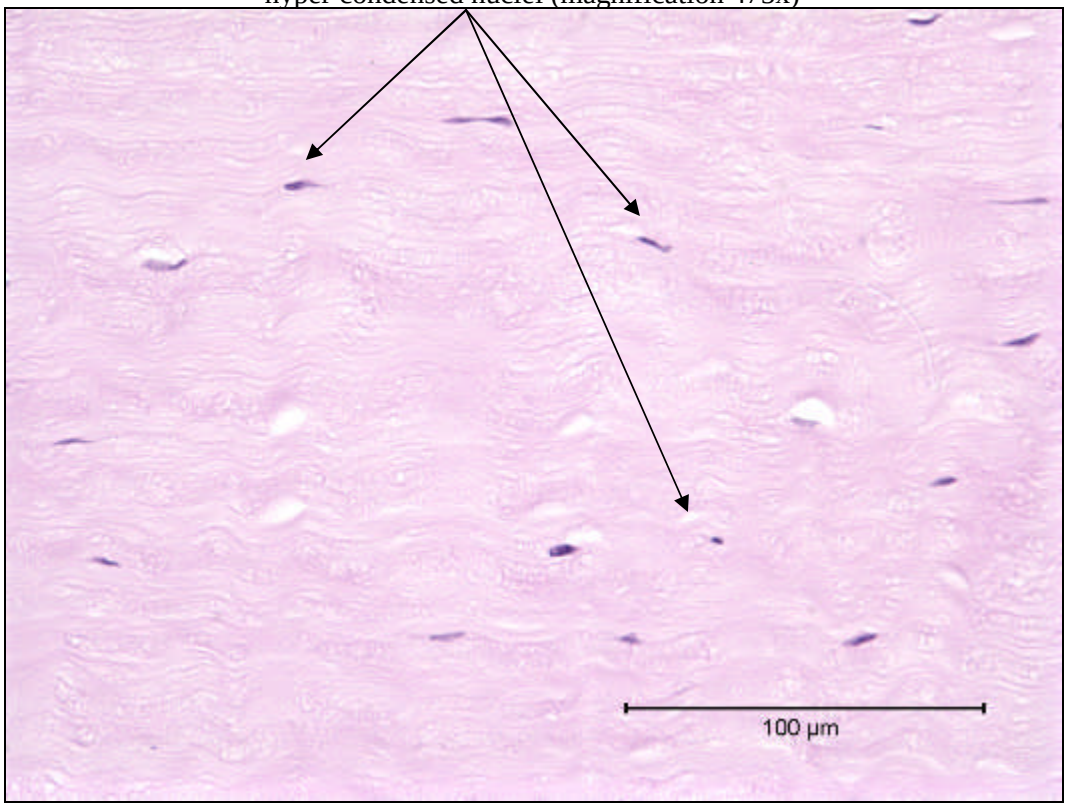


Figure 20. F, neat, 10-minute exposure, 120-minute post-exposure (09/08/05)(cornea #32) - Stroma below mid depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with hyper-condensed nuclei (magnification 475x)

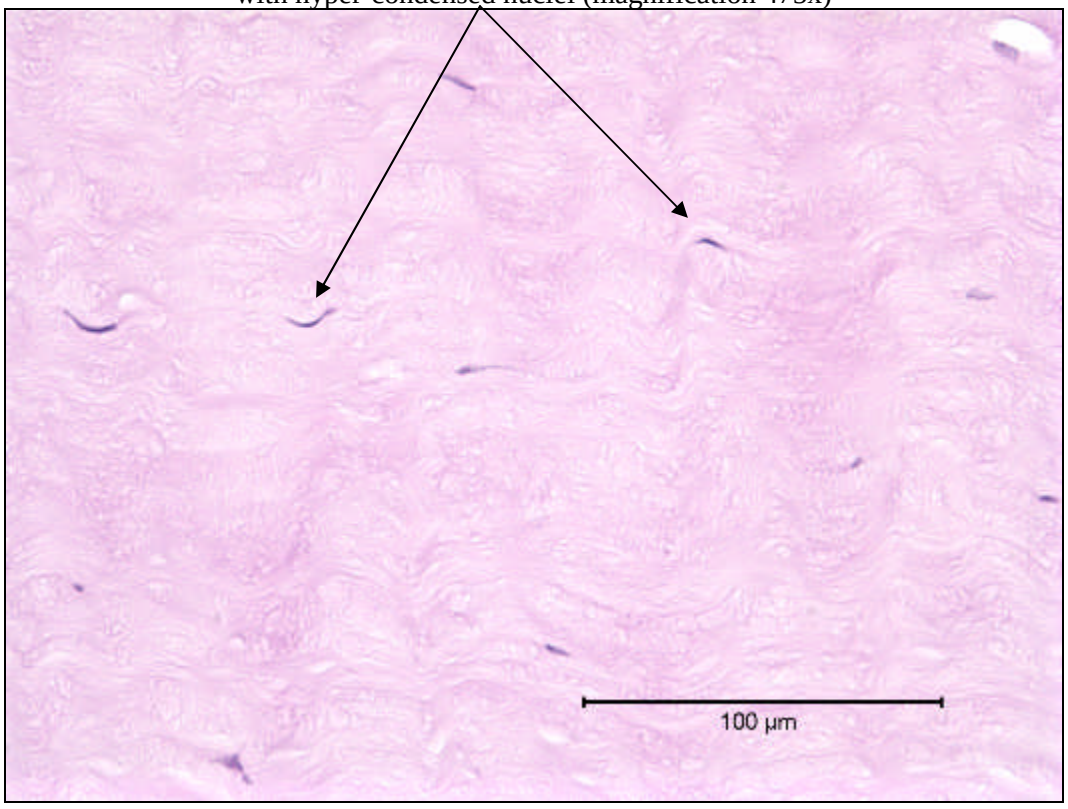


Figure 21. F, neat, 10-minute exposure, 120-minute post-exposure (09/08/05)(cornea #37) - Upper stroma showing the beginning of the gas vacuoles (magnification 237x)

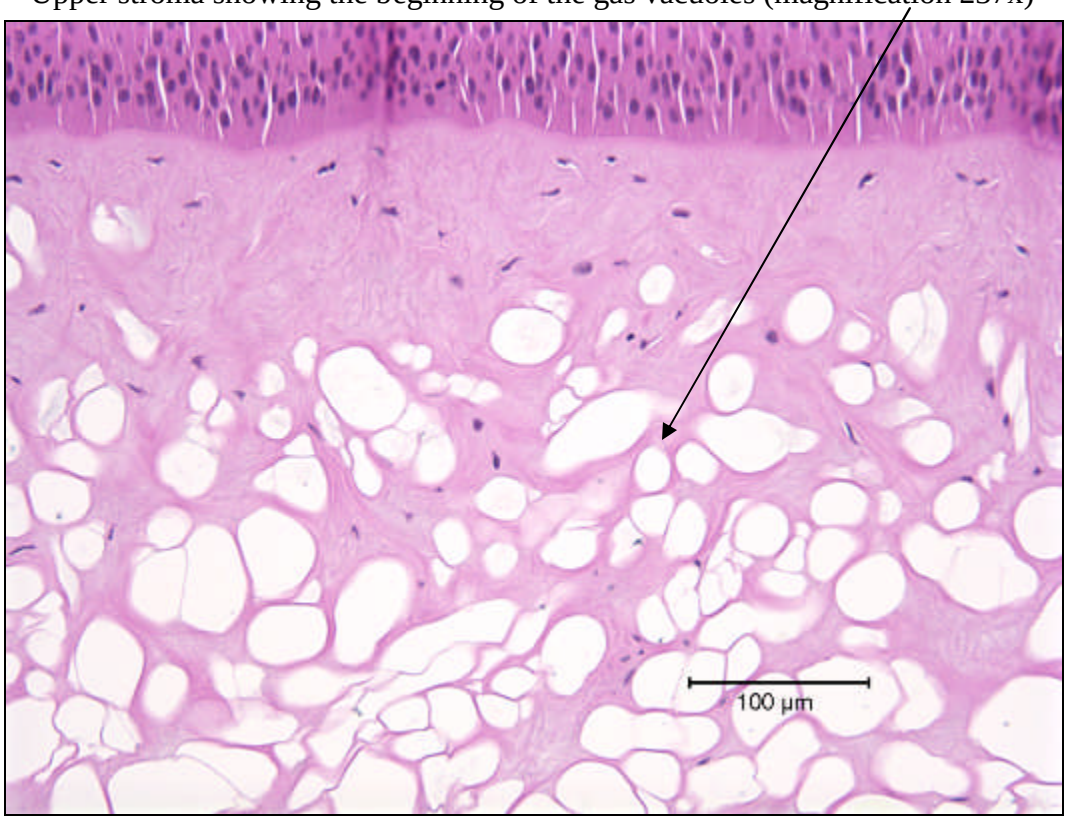


Figure 22. H, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Epithelium (magnification 237x)

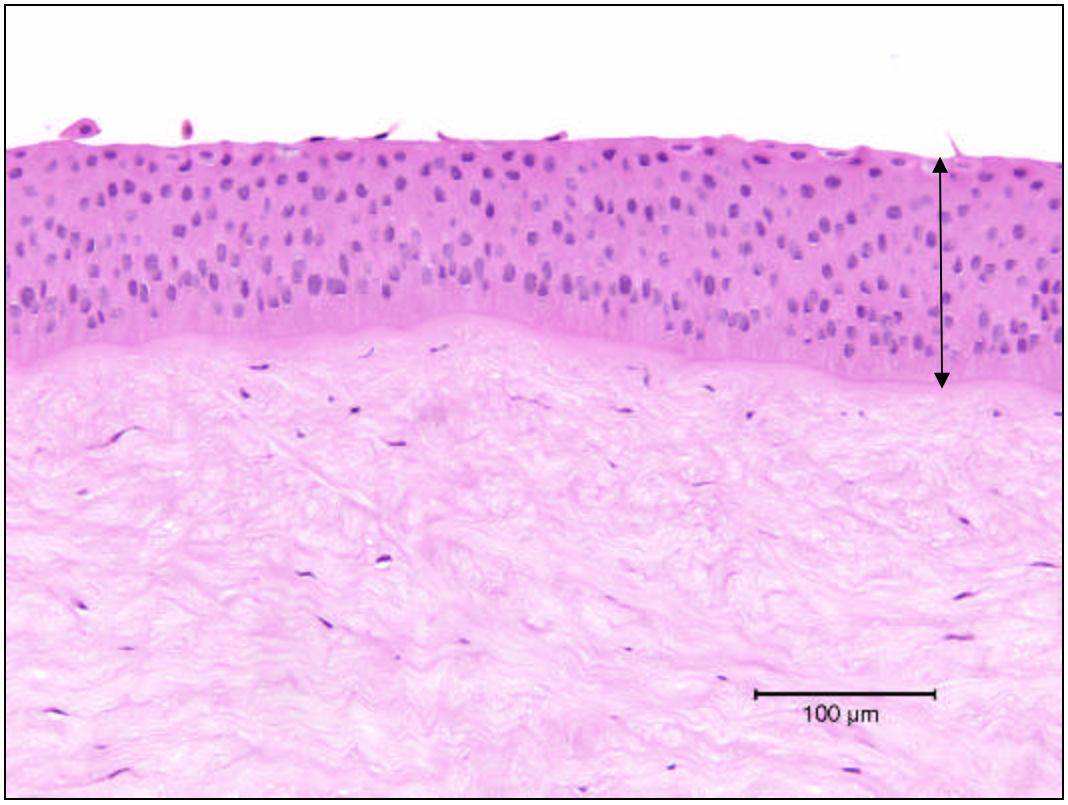


Figure 23. H, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Full thickness (magnification 48x)

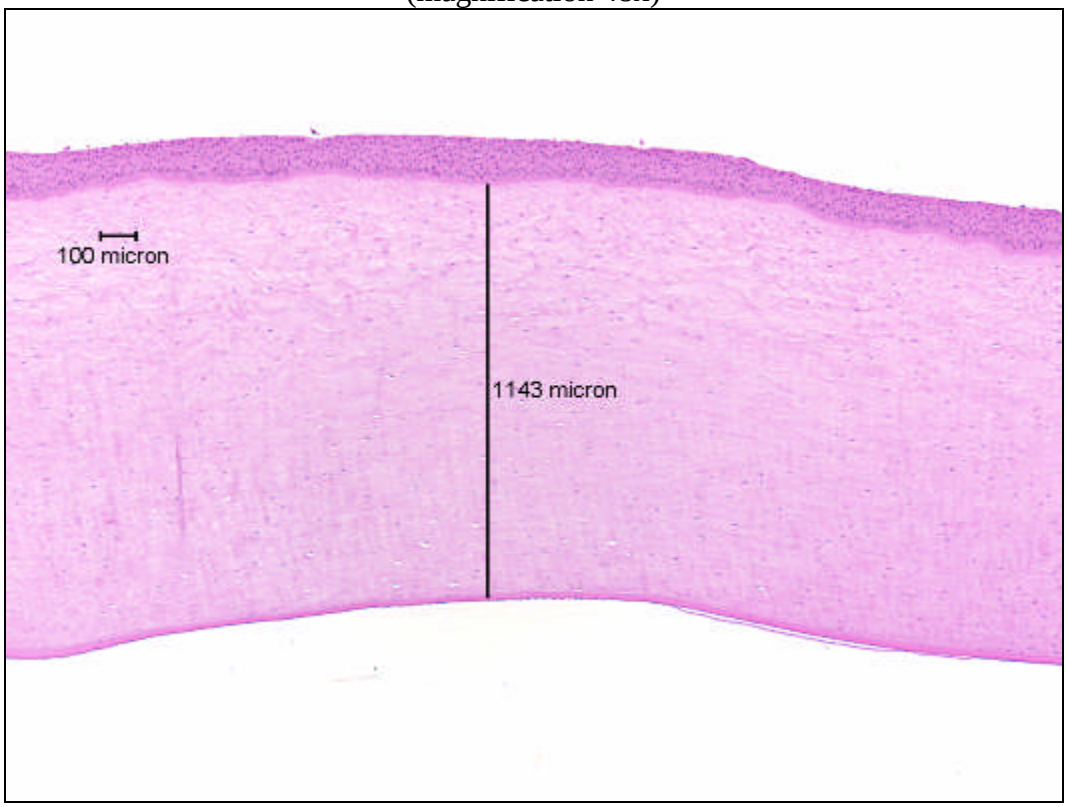


Figure 24. H, neat, 3-minute exposure, 120-minute post-exposure (09/08/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a moderate increase in the frequency of keratocytes with some abnormal nuclear morphology and cytoplasmic eosinophilia (magnification 475x)

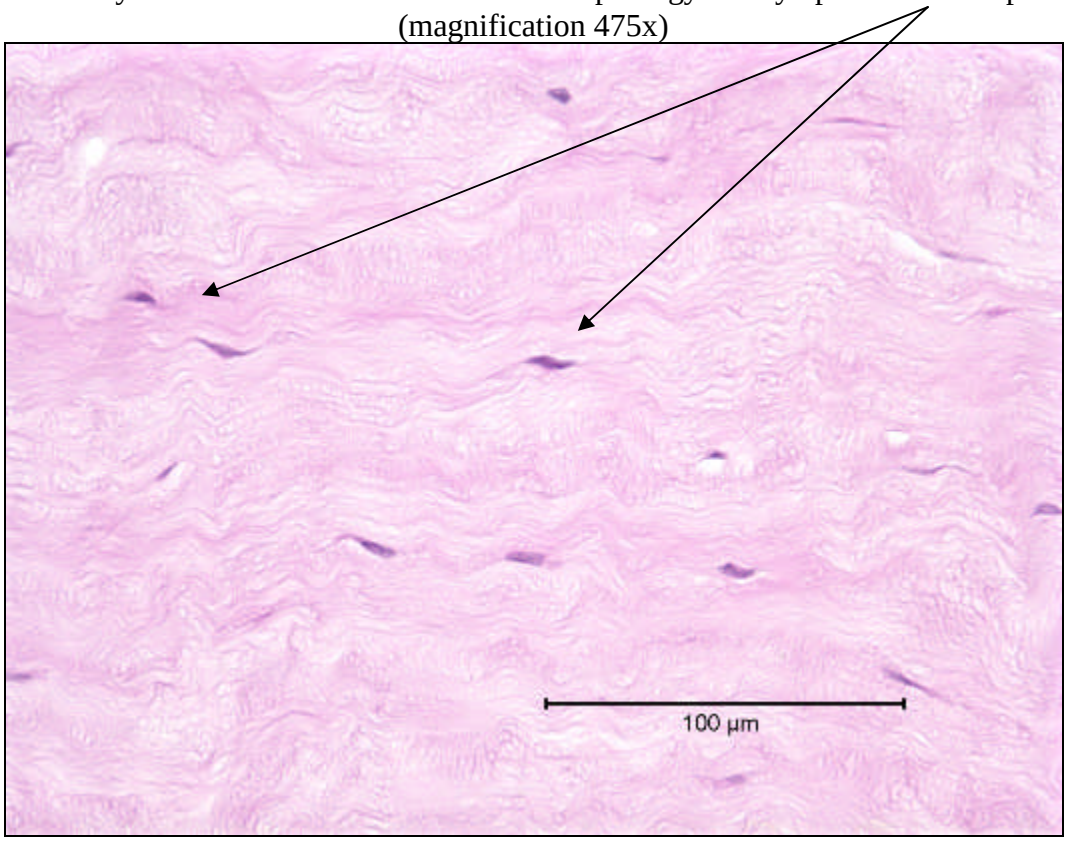


Figure 25. H, neat, 10-minute exposure, 120-minute post-exposure (09/08/05) - Epithelium (overview) (magnification 237x)

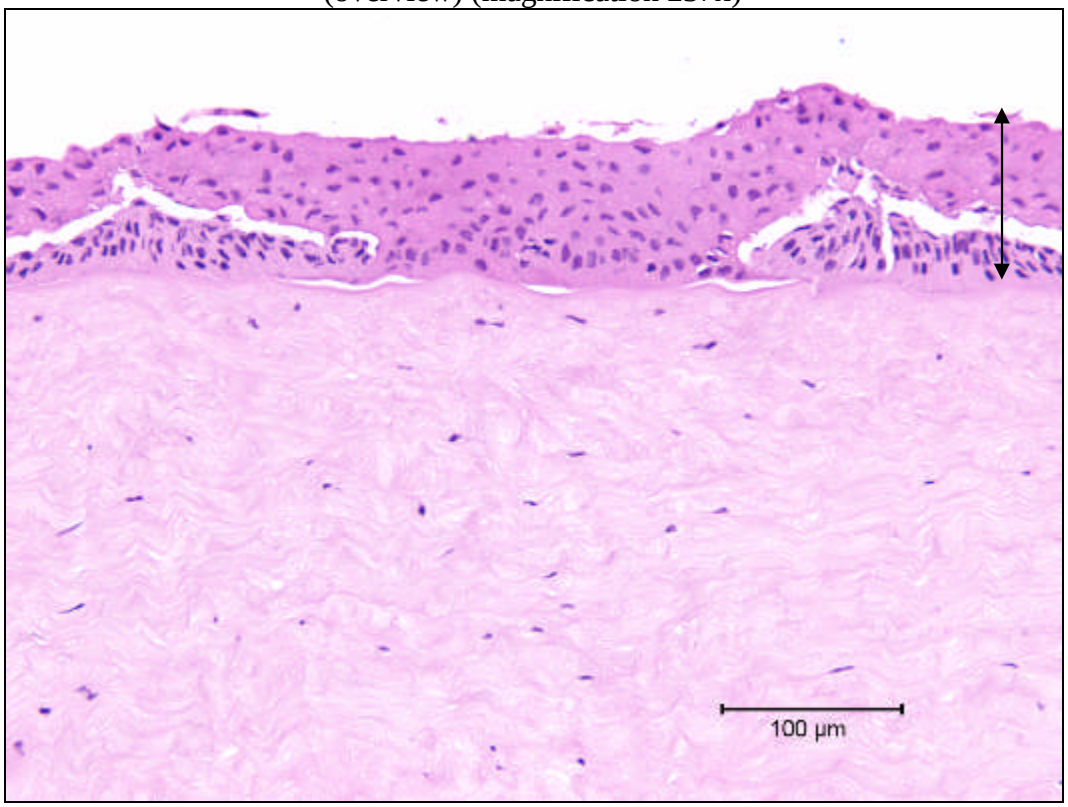


Figure 26. H, neat, 10-minute exposure, 120-minute post-exposure (09/08/05) - Epithelium showing the loss of the squamous cell layer, nuclear and cytoplasmic changes in the wing cells and marked degradation of the basal cells (magnification 475x)

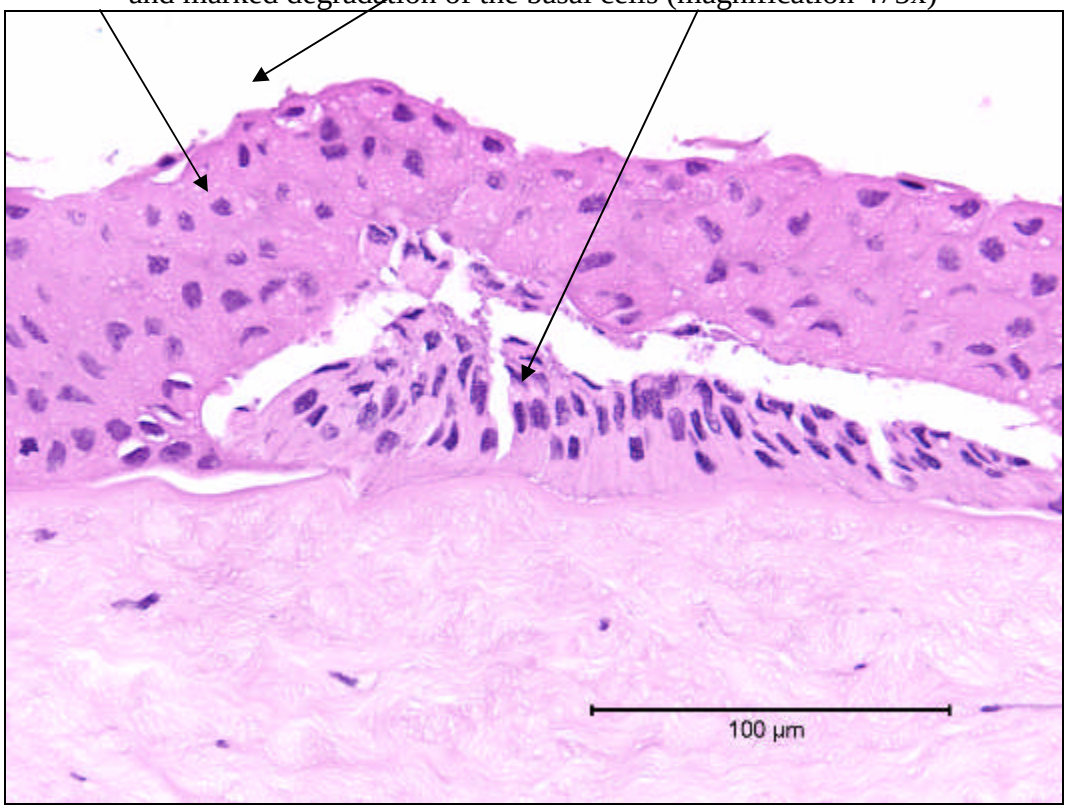


Figure 27. H, neat, 10-minute exposure, 120-minute post-exposure (09/08/05) - Full thickness (magnification 48x)

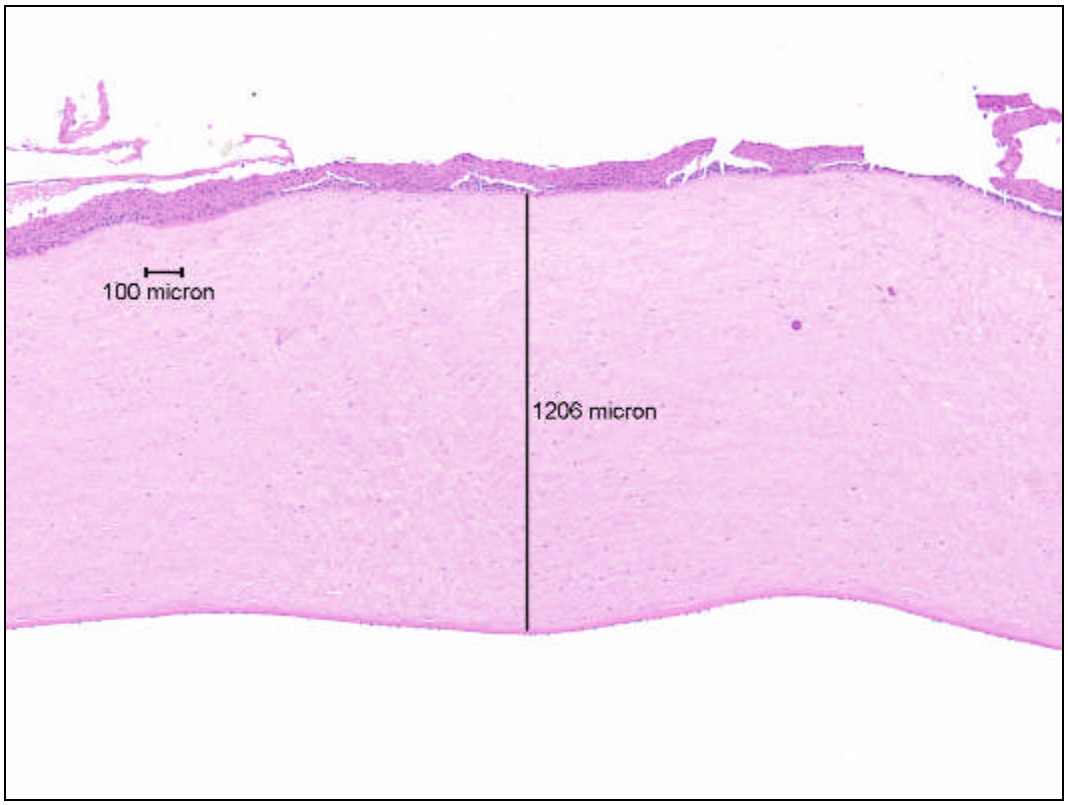


Figure 28. H, neat, 10-minute exposure, 120-minute post-exposure (09/08/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with hyper-condensed nuclei (magnification 475x)

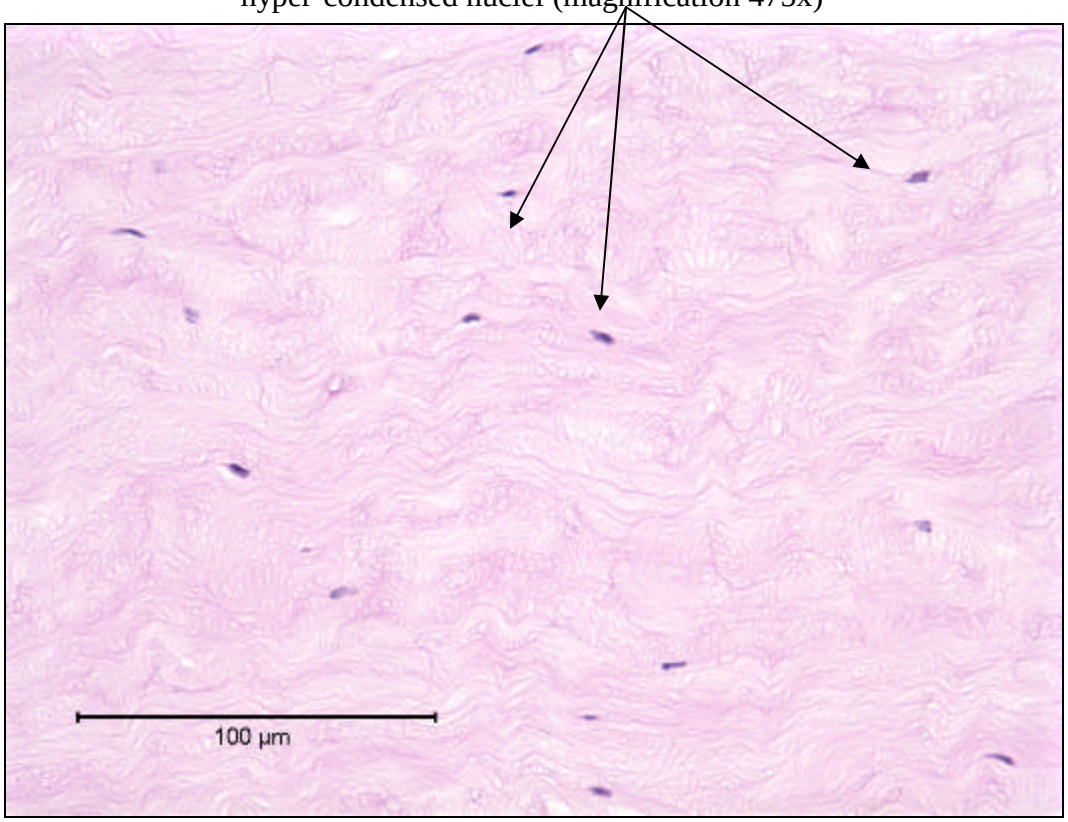
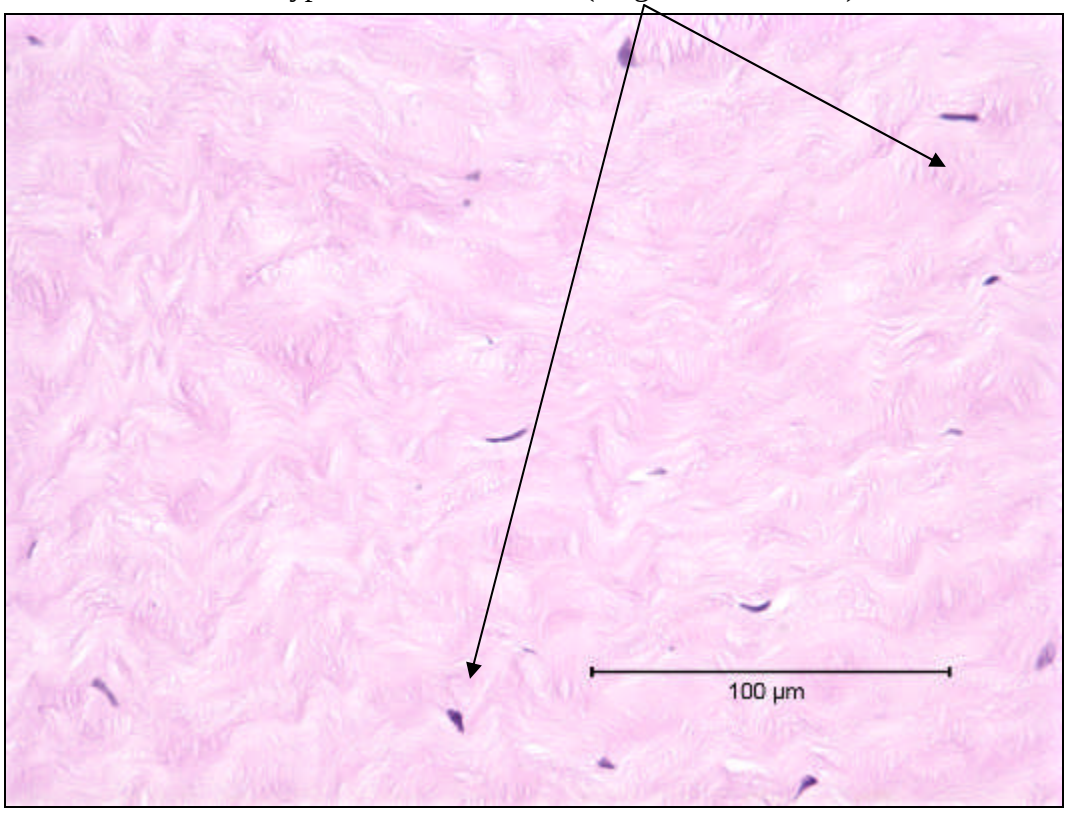


Figure 29. H, neat, 10-minute exposure, 120-minute post-exposure (09/08/05) - Stroma below mid depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with hyper-condensed nuclei (magnification 475x)



APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG.	STDEV
05AE44	8	3	95	92	91.7		
Neat	9	3	94	91	90.7		
3 minutes	10	3	91	88	87.7	90.0	2.1
05AE44	11	2	141	139	138.7		
Neat	12	4	140	136	135.7		
10 minutes	13	4	146	142	141.7	138.7	3.0
05AE45	14	4	7	3	2.7		
Neat	15	4	6	2	1.7		
3 minutes	16	3	5	2	1.7	2.0	0.6
05AE45	17	3	7	4	3.7		
Neat	18	3	9	6	5.7		
10 minutes	19	3	8	5	4.7	4.7	1.0
05AE46	20	3	6	3	2.7		
Neat	23	4	7	3	2.7		
3 minutes	25	3	6	3	2.7	2.7	0.0
05AE46	26	2	5	3	2.7		
Neat	27	3	6	3	2.7		
10 minutes	28	4	6	2	1.7	2.3	0.6
05AE47	29	5	23	18	17.7		
Neat	30	3	14	11	10.7		
3 minutes	31	4	18	14	13.7	14.0	3.5
05AE47	32	3	26	23	22.7		
Neat	35	5	519	514	513.7		
10 minutes	37	5	519	514	513.7	350.0	283.5
05AE48	38	4	7	3	2.7		
Neat	40	5	8	3	2.7		
3 minutes	41	4	8	4	3.7	3.0	0.6
05AE48	42	5	14	9	8.7		
Neat	43	4	13	9	8.7		
10 minutes	44	4	14	10	9.7	9.0	0.6
05AE40	45	3	3	0	-0.3		
Neat	46	3	3	0	-0.3		
3 minutes	47	4	5	1	0.7	0.0	0.6
05AE40	48	4	7	3	2.7		
Neat	49	3	6	3	2.7		
10 minutes	50	3	11	8	7.7	4.3	2.9
Neg. Control	1	3	3	0	NA		
Sterile, DI water	2	3	3	0	NA		
10 minutes	3	3	4	1	NA	0.3	
Pos. Control	4	5	35	30	29.7		
Ethanol	6	3	31	28	27.7		
10 minutes	7	3	30	27	26.7	28.0	1.5
	*24	3					
	*51	4					
	*52	3					
	*54	4					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
10 minutes

Cornea #	OD490
1	0.009
2	0.008
3	0.004
Avg.	0.007

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.068	1	1.061
6	1.294	1	1.287
7	0.856	1	0.849
		Avg. =	1.066
		STDEV =	0.219

05AE44
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
8	1.253	1	1.246
9	0.988	1	0.981
10	1.383	1	1.376
		Avg. =	1.201
		STDEV =	0.201

05AE44
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
11	0.953	1	0.946
12	0.939	1	0.932
13	0.831	1	0.824
		Avg. =	0.901
		STDEV =	0.067

05AE45
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
14	0.046	1	0.039
15	0.076	1	0.069
16	0.056	1	0.049
		Avg. =	0.052
		STDEV =	0.015

05AE45
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
17	0.042	1	0.035
18	0.113	1	0.106
19	0.035	1	0.028
		Avg. =	0.056
		STDEV =	0.043

05AE46
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
20	0.028	1	0.021
23	0.024	1	0.017
25	0.011	1	0.004
		Avg. =	0.014
		STDEV =	0.009

05AE46
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
26	0.057	1	0.050
27	0.127	1	0.120
28	0.057	1	0.050
		Avg. =	0.073
		STDEV =	0.040

05AE47
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
29	0.305	1	0.298
30	0.289	1	0.282
31	0.269	1	0.262
		Avg. =	0.281
		STDEV =	0.018

05AE47
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
32	0.412	5	2.053
35	0.051	1	0.044
37	0.008	1	0.001
		Avg. =	0.699
		STDEV =	1.173

05AE48
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
38	0.012	1	0.005
40	0.023	1	0.016
41	0.036	1	0.029
		Avg. =	0.017
		STDEV =	0.012

05AE48
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
42	0.230	1	0.223
43	0.157	1	0.150
44	0.532	1	0.525
		Avg. =	0.299
		STDEV =	0.199

05AE40
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
45	0.140	1	0.133
46	0.170	1	0.163
47	0.129	1	0.122
		Avg. =	0.139
		STDEV =	0.021

05AE40
Neat
10 minutes

Cornea #	OD490	Factor	OD490
48	0.408	1	0.401
49	0.790	1	0.783
50	1.006	1	0.999
		Avg. =	0.728
		STDEV =	0.303

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AE44	Neat	3 minutes	90.0	1.201	108.0
05AE44	Neat	10 minutes	138.7	0.901	152.2
05AE45	Neat	3 minutes	2.0	0.052	2.8
05AE45	Neat	10 minutes	4.7	0.056	5.5
05AE46	Neat	3 minutes	2.7	0.014	2.9
05AE46	Neat	10 minutes	2.3	0.073	3.4
05AE47	Neat	3 minutes	14.0	0.281	18.2
05AE47	Neat	10 minutes	350.0	0.699	360.5
05AE48	Neat	3 minutes	3.0	0.017	3.3
05AE48	Neat	10 minutes	9.0	0.299	13.5
05AE40	Neat	3 minutes	0.0	0.139	2.1
05AE40	Neat	10 minutes	4.3	0.728	15.2
Ethanol	Neat	10 minutes	28.0	1.066	44.0

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
05AE49	12	6	103	97	96.7		
Neat	13	5	89	84	83.7		
3 minutes	14	3	92	89	88.7	89.7	6.6
05AE49	15	4	177	173	172.7		
Neat	16	3	172	169	168.7		
10 minutes	19	3	170	167	166.7	169.3	3.1
05AE50	20	5	6	1	0.7		
Neat	24	4	5	1	0.7		
3 minutes	25	4	3	-1	-1.3	0.0	1.2
05AE50	27	3	3	0	-0.3		
Neat	28	4	7	3	2.7		
10 minutes	29	4	7	3	2.7	1.7	1.7
05AE51	41	5	8	3	2.7		
Neat	43	4	6	2	1.7		
3 minutes	44	4	8	4	3.7	2.7	1.0
05AE51	45	4	8	4	3.7		
Neat	47	3	10	7	6.7		
10 minutes	48	4	8	4	3.7	4.7	1.7
05AE40	31	6	6	0	-0.3		
Neat	32	5	4	-1	-1.3		
3 minutes	35	5	4	-1	-1.3	-1.0	0.6
05AE40	37	3	6	3	2.7		
Neat	38	3	5	2	1.7		
10 minutes	40	4	5	1	0.7	1.7	1.0
Neg. Control	3	4	4	0	NA		
Sterile, DI water	4	6	7	1	NA		
10 minutes	6	2	2	0	NA	0.3	
Pos. Control	7	4	34	30	29.7		
Ethanol	10	4	25	21	20.7		
10 minutes	11	3	33	30	29.7	26.7	5.2
	*49	4					
	*51	3					
	*52	3					
	*54	6					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
10 minutes

Cornea #	OD490
3	0.003
4	0.009
6	0.000
Avg.	0.004

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
7	0.855	1	0.851
10	0.355	5	1.771
11	1.342	1	1.338
		Avg. =	1.320
		STDEV =	0.460

05AE49
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
12	1.324	1	1.320
13	1.272	1	1.268
14	0.317	5	1.581
		Avg. =	1.390
		STDEV =	0.168

05AE49
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
15	0.440	5	2.196
16	1.446	1	1.442
19	0.349	5	1.741
		Avg. =	1.793
		STDEV =	0.380

05AE50
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
20	0.005	1	0.001
24	0.007	1	0.003
25	0.005	1	0.001
		Avg. =	0.002
		STDEV =	0.001

05AE50
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
27	0.012	1	0.008
28	0.016	1	0.012
29	0.013	1	0.009
		Avg. =	0.010
		STDEV =	0.002

05AE51
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
41	0.069	1	0.065
43	0.034	1	0.030
44	0.074	1	0.070
		Avg. =	0.055
		STDEV =	0.022

05AE51
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
45	0.078	1	0.074
47	0.077	1	0.073
48	0.071	1	0.067
		Avg. =	0.071
		STDEV =	0.004

05AE40
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
31	0.297	1	0.293
32	0.230	1	0.226
35	0.178	1	0.174
		Avg. =	0.231
		STDEV =	0.060

05AE40
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
37	0.702	1	0.698
38	0.677	1	0.673
40	0.906	1	0.902
		Avg. =	0.758
		STDEV =	0.126

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AE49	Neat	3 minutes	89.7	1.390	110.5
05AE49	Neat	10 minutes	169.3	1.793	196.2
05AE50	Neat	3 minutes	0.0	0.002	0.0
05AE50	Neat	10 minutes	1.7	0.010	1.8
05AE51	Neat	3 minutes	2.7	0.055	3.5
05AE51	Neat	10 minutes	4.7	0.071	5.7
05AE40	Neat	3 minutes	-1.0	0.231	2.5
05AE40	Neat	10 minutes	1.7	0.758	13.0
Ethanol	Neat	10 minutes	26.7	1.320	46.5